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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

**TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371**

025219-342

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)

Unassigned **09/914221**INTERNATIONAL APPLICATION NO.
PCT/FR00/00427INTERNATIONAL FILING DATE
February 21, 2000PRIORITY DATE CLAIMED
February 22, 1999

TITLE OF INVENTION

**PROCESS FOR MANUFACTURING MORPHOLINO-NUCLEOTIDES, AND USE THEREOF FOR THE ANALYSIS OF
AND LABELLING OF NUCLEIC ACID SEQUENCES**

APPLICANT(S) FOR DO/EO/US

Florence MARCIAQ; Sylvie SAUVAIGO; Jean-Francois MOURET; Jean-Paul ISSARTEL; Didier MOLKO

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and the PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☒ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

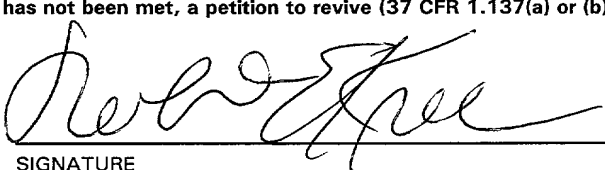
11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

PCT Request, International Search Report, and Cited References

U.S. APPLICATION NO. (If known, use 37 CFR 1.450)
Unassigned

09/914221

INTERNATIONAL APPLICATION NO.
PCT/FR00/00427ATTORNEY'S DOCKET NUMBER
025219-342

				CALCULATIONS	PTO USE ONLY
17. <input checked="" type="checkbox"/> The following fees are submitted:					
Basic National Fee (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1,000.00 (960) International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00 (970) International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00 (958) International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00 (956) International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00 (962) ENTER APPROPRIATE BASIC FEE AMOUNT =					
Surcharge of \$130.00 (154) for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492(e)). 20 <input type="checkbox"/> 30 <input type="checkbox"/>				\$	
Claims	Number Filed	Number Extra	Rate		
Total Claims	17 -20 =	0	X\$18.00 (966)	\$	
Independent Claims	9 -3 =	6	X\$80.00 (964)	\$	480.00
Multiple dependent claim(s) (if applicable)			+ \$270.00 (968)	\$	
TOTAL OF ABOVE CALCULATIONS =				\$	1,340.00
Reduction for 1/2 for filing by small entity, if applicable (see below).				\$	-
SUBTOTAL =				\$	1,340.00
Processing fee of \$130.00 (156) for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492(f)). 20 <input type="checkbox"/> 30 <input type="checkbox"/>				\$	
TOTAL NATIONAL FEE =				\$	1,340.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 (581) per property +				\$	40.00
TOTAL FEES ENCLOSED =				\$	1,380.00
				Amount to be: refunded	\$
				charged	\$
a. <input type="checkbox"/> Small entity status is hereby claimed.					
b. <input checked="" type="checkbox"/> A check in the amount of \$ 1,380.00 to cover the above fees is enclosed.					
c. <input type="checkbox"/> Please charge my Deposit Account No. 02-4800 in the amount of \$ to cover the above fees. A duplicate copy of this sheet is enclosed.					
d. <input type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-4800. A duplicate copy of this sheet is enclosed.					
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO:					
Robert E. Krebs BURNS, DOANE, SWECKER & MATHIS, L.L.P. P.O. Box 1404 Alexandria, Virginia 22313-1404 (650)622-2300					
					
SIGNATURE					
Robert E. Krebs					
NAME					
25,885					
REGISTRATION NUMBER					

09/914221
JC05 Rec'd PCT/PTO 22 AUG 2001

Patent
Attorney's Docket No. 025219-342

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of)
)
 Marciaq, et al.) Group Art Unit: Unassigned
)
 Application No.: Unassigned) Examiner: Unassigned
)
 Filed: Herewith)
)
 For: PROCESS FOR MANUFACTURING)
 MORPHOLINO-NUCLEOTIDES, AND)
 USE THEREOF FOR THE ANALYSIS)
 OF AND LABELLING OF NUCLEIC)
 ACID SEQUENCES)

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination, please amend the subject application as follows:

IN THE SPECIFICATION

Please amend the specification by inserting before the first line the sentence:

"This application is a national phase of PCT/FR00/00427, and International Applications Nos. 99/02170 and 99/12001, which was filed on February 22, 1999 and September 27, 1999 respectively, and was not published in English."

IN THE CLAIMS:

Please amend claim 4 as follows:

4. (Amended) Process according to Claim 1, in which the enzyme is the Klenow fragment of DNA polymerase.

Please amend claim 5 as follows:

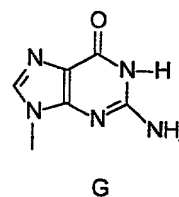
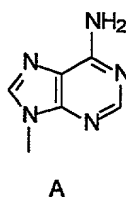
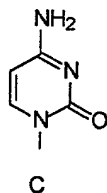
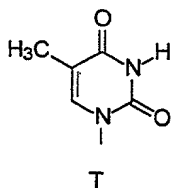
5. (Amended) Process according to Claim 1, in which the enzyme is a heat-resistant polymerase of a *Thermophilus* bacterium or terminal transferase or reverse transcriptase.

Please amend claim 6 as follows:

6. (Amended) Process according to Claim 1 in which the nucleic base is a natural nucleic base chosen from adenine, guanine, cytosine, thymine, uracil, xanthine, hypoxanthine and 2-aminopurine, and derivatives thereof.

Please amend claim 7 as follows:

7. (Amended) Process according to Claim 1 in which R¹ corresponds to one of the following formulae:



Please amend claim 8 as follows:

8. (Amended) Process according to Claim 1, in which the label is chosen from radioactive products, luminescent products, electroluminescent and fluorescent products, molecules capable of coupling with other molecules, molecules which allow interactions of the antigen-antibody type, and enzymatic labels.

Please amend claim 9 as follows:

9. (Amended) Process according to Claim 1, in which R³ is a fluorophore.

Please amend claim 11 as follows:

11. (Amended) Process according to Claim 1, in which the derivative, the modified morpholino-nucleotide or the chain terminator is compound (I) in monophosphate form.

PLEASE ADD THE FOLLOWING CLAIMS:

18. Process according to Claim 2, in which the enzyme is the Klenow fragment of DNA polymerase.

19. Process according to Claim 3, in which the enzyme is the Klenow fragment of DNA polymerase.

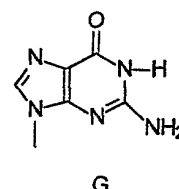
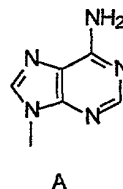
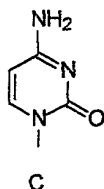
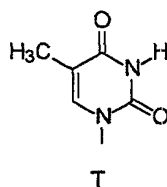
20. Process according to Claim 2, in which the enzyme is a heat-resistant polymerase of a *Thermophilus* bacterium or terminal transferase or reverse transcriptase.

21. Process according to Claim 3, in which the enzyme is a heat-resistant polymerase of a *Thermophilus* bacterium or terminal transferase or reverse transcriptase.

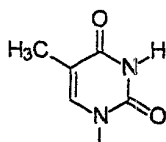
22. Process according to Claim 2 in which the nucleic base is a natural nucleic base chosen from adenine, guanine, cytosine, thymine, uracil, xanthine, hypoxanthine and 2-aminopurine, and derivatives thereof.

23. Process according to Claim 3 in which the nucleic base is a natural nucleic base chosen from adenine, guanine, cytosine, thymine, uracil, xanthine, hypoxanthine and 2-aminopurine, and derivatives thereof.

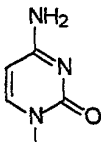
24. Process according to Claim 2 in which R¹ corresponds to one of the following formulae:



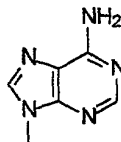
25. Process according to Claim 3 in which R¹ corresponds to one of the following formulae:



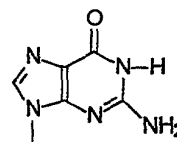
T



C



A



G

26. Process according to Claim 2, in which the label is chosen from radioactive products, luminescent products, electroluminescent and fluorescent products, molecules capable of coupling with other molecules, molecules which allow interactions of the antigen-antibody type, and enzymatic labels.

27. Process according to Claim 3, in which the label is chosen from radioactive products, luminescent products, electroluminescent and fluorescent products, molecules capable of coupling with other molecules, molecules which allow interactions of the antigen-antibody type, and enzymatic labels.

28. Process according to Claim 2, in which R³ is a fluorophore.

29. Process according to Claim 3, in which R³ is a fluorophore.

30. Process according to Claim 28, in which R³ is chosen from fluorescein derivatives, biotin derivatives and rhodamine derivatives.

31. Process according to Claim 29, in which R^3 is chosen from fluorescein derivatives, biotin derivatives and rhodamine derivatives.

32. Process according to Claim 2, in which the derivative, the modified morpholino-nucleotide or the chain terminator is compound (I) in monophosphate form.

33. Process according to Claim 3, in which the derivative, the modified morpholino-nucleotide or the chain terminator is compound (I) in monophosphate form.

REMARKS

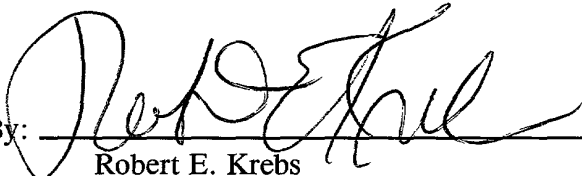
Entry of the foregoing amendment to the Specification is requested to comply with the requirements of 37 C.F.R. 1.78(a)(2).

The claims of the subject application have been amended to avoid multiple dependency. Favorable consideration of the subject application is respectfully requested.

Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached page is captioned "Version with markings to show changes made."

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

By: 
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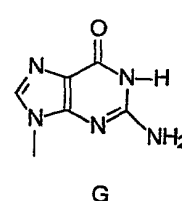
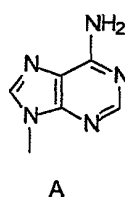
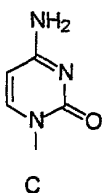
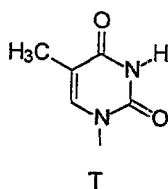
Date: August 21, 2001

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the claims:

Claim 4, 5, 6, 7, 8, 9, and 11 have been amended as follows:

4. Process according to Claim 1, ~~2 or 3~~, in which the enzyme is the Klenow fragment of DNA polymerase.
5. Process according to Claim 1, ~~2 or 3~~, in which the enzyme is a heat-resistant polymerase of a *Thermophilus* bacterium or terminal transferase or reverse transcriptase.
6. Process according to ~~one of~~ Claims 1 ~~to 5~~, in which the nucleic base is a natural nucleic base chosen from adenine, guanine, cytosine, thymine, uracil, xanthine, hypoxanthine and 2-aminopurine, and derivatives thereof.
7. Process according to ~~any one of~~ Claims 1 ~~to 5~~, in which R¹ corresponds to one of the following formulae:



8. Process according to ~~one of~~ Claims 1 ~~to 7~~, in which the label is chosen from radioactive products, luminescent products, electroluminescent and fluorescent products, molecules capable of coupling with other molecules, molecules which allow interactions of the antigen-antibody type, and enzymatic labels.
9. Process according to ~~any one of the~~ claims 1 ~~to 7~~, in which R³ is a fluorophore.
11. Process according to Claim 1, ~~2 or 3~~, in which the derivative, the modified morpholino-nucleotide or the chain terminator is compound (I) in monophosphate form.

Claims 18 - 33 have been added.



DEMANDE INTERNATIONALE PUBLIÉE EN VERTU DU TRAITE DE COOPERATION EN MATIÈRE DE BREVETS (PCT)

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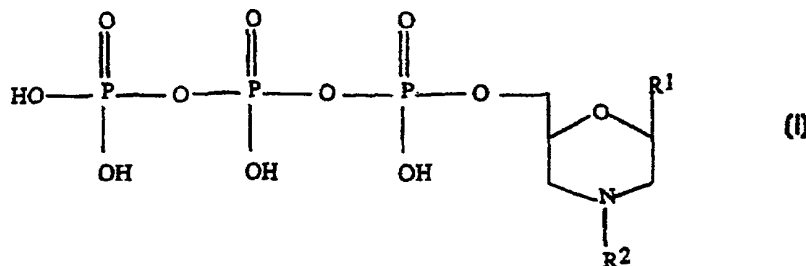
(81) Etats désignés: CA, JP, US, brevet européen (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Publiée

Avec rapport de recherche internationale.
Avant l'expiration du délai prévu pour la modification des revendications, sera republiée si des modifications sont reçues.

(54) Title: METHODS FOR MAKING MORPHOLINO-NUCLEOTIDES, AND THEIR USE FOR ANALYSING AND MARKING NUCLEIC ACID SEQUENCES

(54) Titre: PROCEDE DE FABRICATION DE MORPHOLINO-NUCLEOTIDES, ET LEUR UTILISATION POUR L'ANALYSE ET LE MARQUAGE DE SEQUENCES D'ACIDES NUCLEIQUES



(I)

(57) Abstract

The invention concerns the use of morpholino-nucleosides of formula (I) wherein: R¹ represents a nucleic base and R² represents a group corresponding to the following formulae: -(CH₂)_n-NH₂, -(CH₂)_n-SH, -(CH₂)_n-COOH, -(CH₂)_n-OH, -(CH₂)_n-NH-R³, -(CH₂)_n-SR³, -(CH₂)_n-CO-R³, -(CH₂)_n-OR³ wherein: n is an integer ranging from 1 to 12 and R³ is a group derived from a marker, a protein, an enzyme, a fatty acid or a peptide, as chain terminators in a DNA or RNA sequencing process by Sanger method, or for marking DNA or RNA fragments.

(57) Abrégé

L'invention concerne l'utilisation de morpholino-nucléosides de formule (I): dans laquelle R¹ représente une base nucléique et R² représente un groupe répondant à l'une des formules suivantes: -(CH₂)_n-NH₂, -(CH₂)_n-SH, -(CH₂)_n-COOH, -(CH₂)_n-OH, -(CH₂)_n-NH-R³, -(CH₂)_n-S-R³, -(CH₂)_n-CO-R³, -(CH₂)_n-OR³ dans lesquelles n est un nombre entier allant de 1 à 12 et R³ est un groupe dérivé d'un marqueur, d'une protéine, d'une enzyme, d'un acide gras ou d'un peptide, comme terminateurs de chaînes dans un procédé de séquençage d'ADN ou d'ARN par la méthode de Sanger, ou pour le marquage de fragments d'ADN ou d'ARN.

3/PRTS

09/914221
JC05 Rec'd PCT/PTO 22 AUG 2001

1

PROCESS FOR MANUFACTURING MORPHOLINO-
NUCLEOTIDES, AND USE THEREOF FOR THE ANALYSIS OF AND
LABELLING OF NUCLEIC ACID SEQUENCES

DESCRIPTION

5 **Technical field**

The present invention relates to the manufacture of nucleic acid (DNA or RNA) fragments enzymatically extended with morpholino-nucleoside triphosphates. This elongation may be used for the analysis of nucleic acid sequences by incorporating these derivatives into nucleic acid chains, and also the enzymatic labelling and immobilization or detection of sequences.

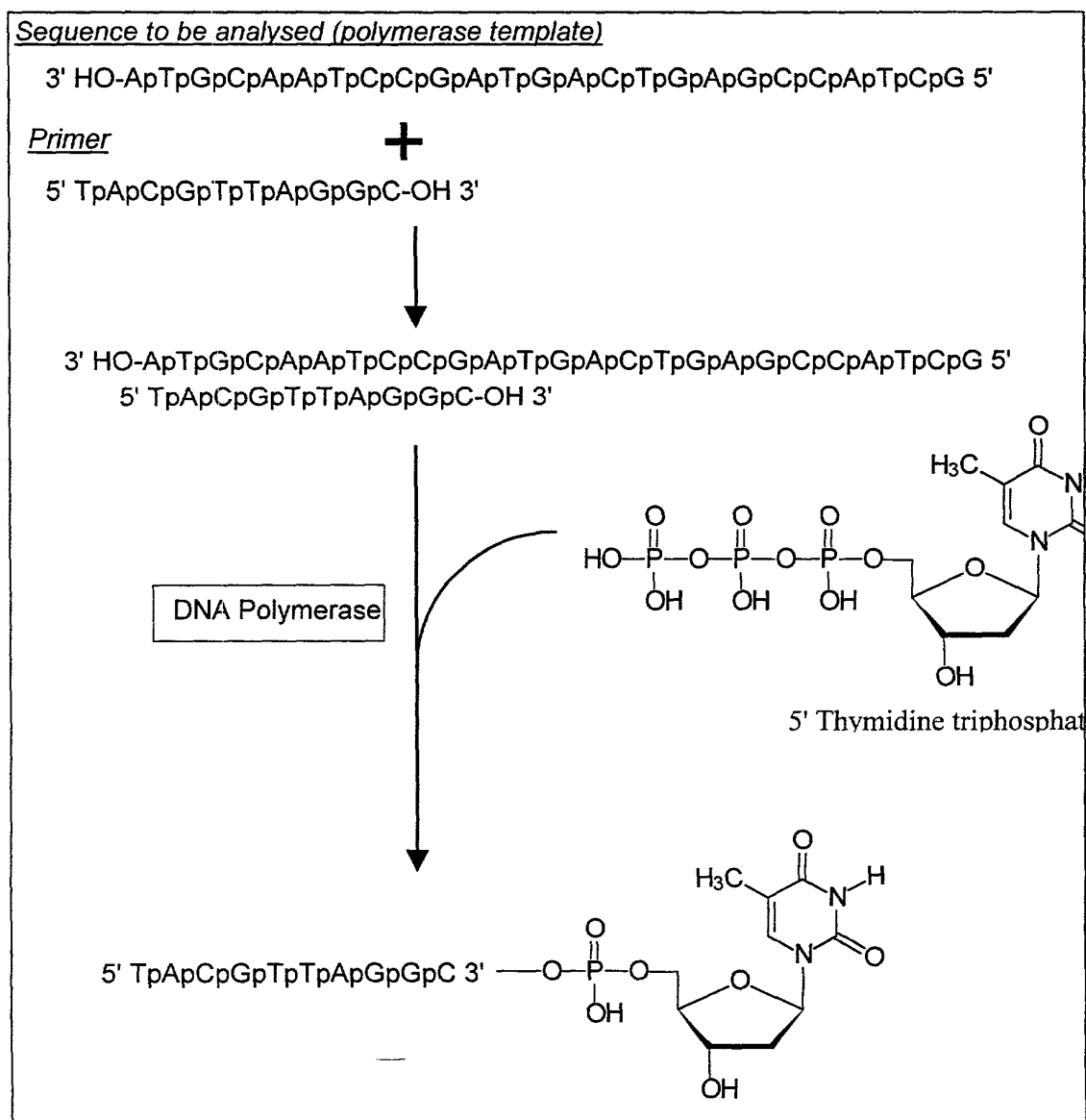
These morpholino-nucleoside triphosphates may also be used with an additional molecule which may have various roles in many applications.

Prior art

The method most widely used for analysing nucleic acid sequences is the enzymatic "chain termination" technique, developed by Sanger et al. in Proceedings of National Academy of Science, 74, 1977, p. 5463-5467 [1]. It is based on the properties of DNA-dependent DNA polymerases to create DNA polymers complementary to the sequence of a DNA strand serving as a template, from a mixture of natural nucleoside triphosphate monomers. The process consists, starting with the DNA strand to be analysed, in making a series of

2
copies of the complementary strand by adding to the
conventional reaction medium molecules known as "chain
terminators" and then analysing the length of the newly
5 template. The principle of the method is explained in
Table 1 below.

Table 1



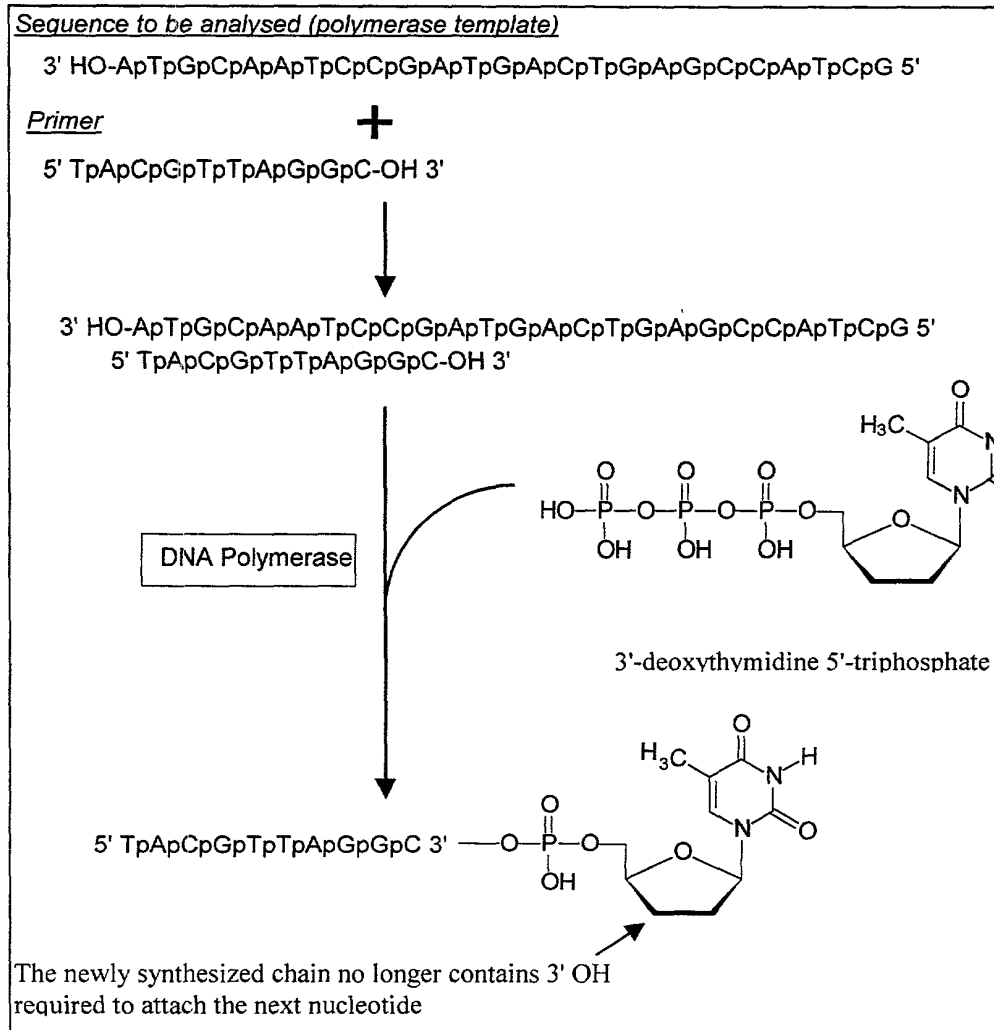
10 This Table 1 illustrates what happens when the
DNA polymerase, a primer consisting of a small

oligonucleotide, generally of less than 25 bases, and the mixture of the four natural nucleoside triphosphates are placed in contact with the DNA strand whose sequence it is desired to determine, which constitutes the template.

5 The primer corresponds to the start of the complementary sequence of the DNA strand to be analysed. Starting with this primer, which interacts spontaneously with the complementary sequence of the DNA strand to be analysed (hybridization), the enzyme incorporates nucleotides
10 complementary to the template to construct by elongation-polymerization a new DNA strand, which is a copy complementary to the said template. The new nucleotides are incorporated exclusively from the 3'-OH terminal end of the growing chain, sequentially and in compliance with
15 the Watson & Crick rules of complementarity between bases. A thymine is incorporated into the newly formed strand by complementarity with an adenine present in the strand serving as the template, a guanine is incorporated in complementarity with a cytosine, and vice versa. If
20 all the required compounds are supplied in unlimited amount, the enzyme catalyzes the polymerization of the strand formed until said strand represents the entire strand complementary to the matrix.

On the other hand, if a molecule which is
25 recognized by the polymerase but which has no free 3'-OH terminal end is added to the reaction medium, each time this molecule is incorporated, the polymerization work of the enzyme will be interrupted because the chain can no longer grow on account of the absence of a site available
30 to attach a new nucleotide (creation of interrupted newly-formed strands). This is illustrated in Table 2 below with 3'-deoxythymidine 5'-triphosphate.

Table 2



5

Using this thymidine derivative which will be referred to as a "T chain terminator" at a (inaudible) concentration, a series of DNA strands whose size is randomly fixed by the position of the adenines in the template is obtained for a given template. The result obtained is illustrated in Table 3. The sequence of the

10 template is written in the first line and the sequence of

5

the newly formed strands created with the T chain terminator (noted S) is written in the following lines.

Table 3

TEMPLATE

3'- A T G C A T T C C G A C C T C T G A T C A G -5'

COPIES OF THE TEMPLATE

5'- S

5'- T A C G S

5'- T A C G T A A G G C S

5'- T A C G T A A G G C T G G A G A C S

5'- T A C G T A A G G C T G G A G A C T A G S

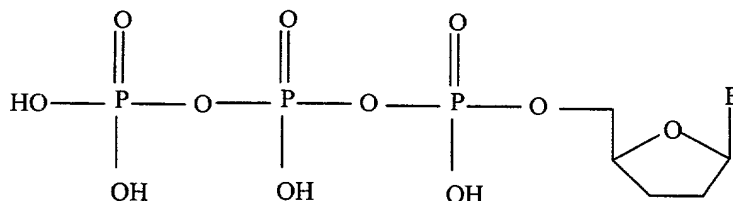
In this example, the template comprises 5 adenines in the region which is detailed, and the DNA polymerase may thus produce 5 interrupted newly formed strands, of different lengths.

It then suffices to analyse this mixture by polyacrylamide gel electrophoresis in denaturing medium to determine the length of each of the strands obtained using the T chain terminator. The size of the interrupted newly formed strands makes it possible to deduce the position of the adenines on the matrix.

By repeating this experiment three times with A, G and C chain terminator products, respectively, four series of DNA fragments are obtained in total, the length of which fragments makes it possible to determine the entire sequence of the template strand.

The technique of RNA sequencing is based on the same principles, the difference being that the enzyme used is a reverse transcriptase (or RNA-dependent DNA polymerase).

The products most commonly used as chain terminators to stop the action of the DNA polymerases are 2',3'-dideoxynucleoside triphosphates of formula:



in which B represents one of the nucleic bases A, C, G or T, as described in document [1].

5 The structure of these products compared with that of the natural nucleoside triphosphates shows the absence of the hydroxyl function in the 3' position which serves as the position of attachment for the next nucleotide.

10 The chemical synthesis of 2',3'-dideoxynucleotides is performed according to a long and laborious protocol comprising three major steps. In the case of guanine, the first step of this process is the protection of the exocyclic amine function of the guanine and of the primary 5' hydroxyl function of the sugar. The
15 3' hydroxyl function is then deleted, by removal and then by reduction of the 2'-3' double bond generated. The final step is the preparation of the triphosphate derivative.

20 Other chain terminators have been described in document WO-A-96/23807 [2]. These are the 5'-triphosphates of arabinonucleosides, of 3'-fluoro-2',3'-dideoxynucleosides, 3'-azido-2',3'-dideoxynucleosides or 3'-amino-2',3'-dideoxynucleosides. These are also
25 laborious to synthesize.

 Originally in the Sanger method, the visualization of the DNA fragments synthesized was achieved by radioactive labelling with ³²P at the 5' end of the primer used to initiate the polymerization of the

complementary strand. A modification was made by using primers bearing a fluorophore. This improvement has a bearing only on the ease of use, since it dispenses with the use of radioactive materials, but it is still
5 necessary to carry out four sequencing reactions, each using a different polymerization terminator (A, G, T or C terminator).

A new landmark was passed with the use of sequence terminators bearing fluorophores on their
10 nucleic base, as described by Prober et al. in Science, 238, 1987, pages 336-341 [3].

Under these conditions, the newly synthesized strands are no longer labelled before the sequencing reaction, but rather directly at the time of
15 incorporation of the sequence terminator. By taking care to select a fluorophore with different optical properties for each DNA base, the experimental protocol was greatly simplified. Only one reaction is performed with the four terminators mixed together. As a result, starting with a
20 single electrophoresis lane, the four nucleotides of the sequence are distinguished by virtue of the different emission wavelengths of the four terminators.

This simplification of the analysis protocol is not without drawbacks. Specifically, the fluorophores
25 are grafted directly onto the base. This structural modification, located in the direct region of the sites of hydrogen bonding governing the recognition between the bases, results in a decrease in the recognition by the enzymes. To compensate for this, an increase in the
30 concentration of the terminators is recommended, which leads to a very great consumption of the starting material having a very high added value. Furthermore,

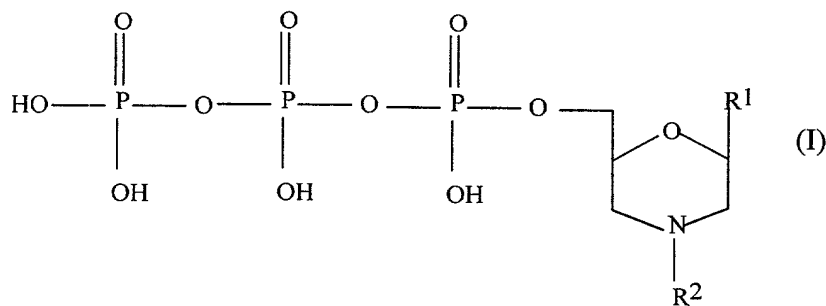
8

these molecules are still just as difficult to synthesize.

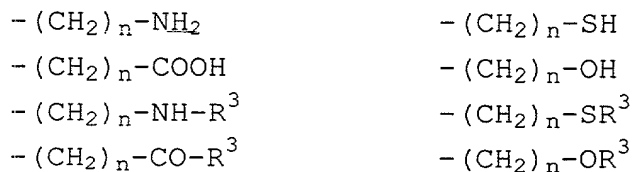
Description of the invention

One subject of the present invention is in particular the use, in a sequencing process of this type, of chain terminators consisting of nucleotide triphosphate analogues which are easier to synthesize and which furthermore make it possible to carry out efficient labelling without modifying the nucleic bases.

Thus, one subject of the invention is a process for sequencing a nucleic acid (DNA or RNA) by the technique of enzymatic polymerization of the sequence complementary to this nucleic acid using chain terminators, in which at least one of the chain terminators has as precursor a compound corresponding to the formula:



in which R^1 represents a nucleic base and R^2 represents a group corresponding to one of the following formulae:



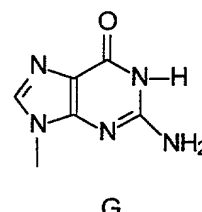
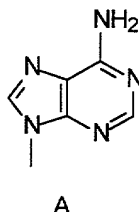
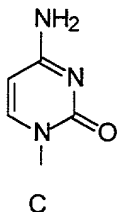
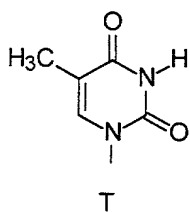
in which n is an integer ranging from 1 to 12 and R^3 is a group derived from a label, a protein, an enzyme, a fatty acid or a peptide.

5 The chain terminators used in this process are nucleotide derivatives comprising a nucleic base R^1 which allows recognition by the polymerases and transcriptases, and compliance with the Watson and Crick rules of complementarity.

10 The nucleic bases used for R^1 may be natural or synthetic. The natural bases are generally chosen from adenine, guanine, cytosine, thymine, uracil, xanthine, hypoxanthine and 2-aminopurine, and derivatives thereof.

15 The synthetic bases are analogues or derivatives of the natural nucleic bases, which are capable of interacting with the natural bases.

 Preferably, R^1 corresponds to one of the following formulae:



20

 In the nucleotide derivatives of formula (I), the saccharide portion is replaced with a suitably substituted morpholine comprising:

25 1°) A hydroxymethyl function close to the ring oxygen, esterified with a triphosphoric acid group. This portion of the molecule mimics the 4',5' portion of

nucleotides and allows binding by the polymerase or the transcriptase to the growing DNA or RNA chain.

2°) An amine function substituted with R², which can optionally allow the grafting of a chromophore or of a biologically active group and, especially, which prevents the attachment of another nucleotide (interruption of the polymerization).

Compared with the derivatives conventionally used in the Sanger method, such as those described in documents [1], [2] and [3], these compounds may be synthesized in a single step directly from ribonucleoside triphosphates, as will be seen below.

The advantage of these compounds lies in the very wide choice of groups R² (substituents of the morpholine ring) which may be used and which allow this ring to be functionalized. Functions such as acids, amines, thiols or ethers may be added and will allow the grafting of varied chemical compounds, in particular of labels that are useful for indentifying DNA or RNA fragments.

The label used for R³ may be chosen from a very large set of molecules known for labelling nucleotides. They may be chosen, for example, from radioactive products, luminescent, electroluminescent and fluorescent products, molecules capable of coupling with other molecules, molecules allowing interactions of antigen-antibody type, and enzymatic labels.

Preferably, for the sequencing of nucleic acids, R³ is a fluorophore chosen, for example, from any fluoroscein or rhodamine derivative. Biotin derivatives may also be used. In particular, derivatives used for labelling nucleic acids will be chosen.

Nucleoside derivatives in which the saccharide portion of the nucleoside has been replaced with a morpholine have already been synthesized in the prior art, as is seen in the following documents:

- 5 - Hileman et al., Bioconjugate Chemistry, 5, 1994, pages 436-444 [4],
- Broker et al., Nucleic Acids Research, 5, 1978, pages 363-385 [5],
- Agrawal et al., Nucleic Acids Research, 14, 1986, pages 6227-6245 [6],
- 10 - FR-A- 2 710 068 [7], and
- Rayford et al., Journal of Biological Chemistry, 260, 1985, pages 15708-15713, [8].

The nucleoside derivatives in document [4] 15 comprise a morpholino ring which is substituted with a fluorescein or a rhodamine. They are used for the study of proteins rather than as chain terminators in a nucleic acid sequencing process.

Their manufacture differs from that of the 20 process reported herein, since the fluorophore is incorporated directly onto the morpholine ring. The technique we are describing involves a step of intermediate purification which allows us to isolate and fully characterize the final product, in contrast with 25 Hileman et al.

Document [5] concerns transfer RNA modified at its 3' end with a nucleoside derivative comprising a morpholine ring substituted with a biotin. This product is used as a chemical label for transfer RNAs to study 30 the chromosomal localization of transfer RNA genes.

Document [6] concerns an oligonucleotide comprising a morpholine ring coupled to a biotin, which

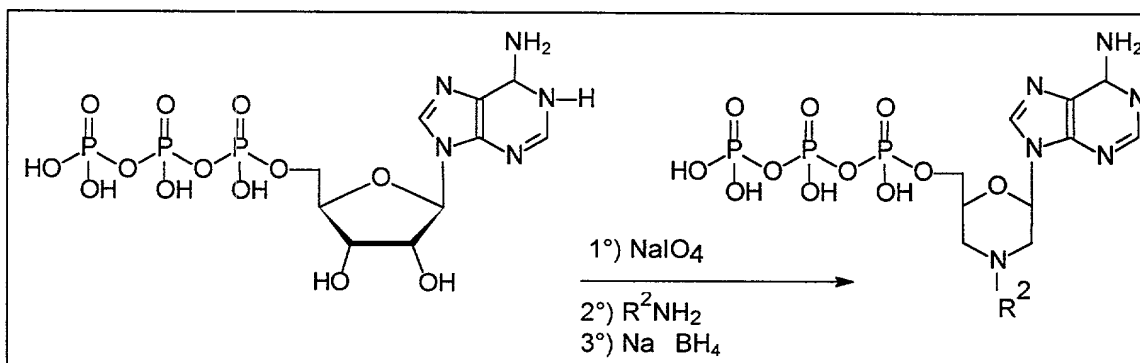
is used as a probe for detecting and isolating specific genes.

Document [7] describes nucleoside derivatives comprising a substituted morpholine ring. They are used for preparing antibodies raised against a hapten bound to the morpholine ring of the nucleoside derivative.

Document [8] illustrates a morpholinoadenosine substituted with CH_2COOH , which is used for affinity chromatography.

Thus, none of these documents concerns the use of nucleotide derivatives such as those of the invention, as chain terminators, in a nucleic acid sequencing process according to the Sanger method.

The nucleotide derivatives used in the process of the invention may be prepared in a single step, directly from ribonucleoside triphosphates, according to the following reaction scheme illustrated with R^1 representing adenine.



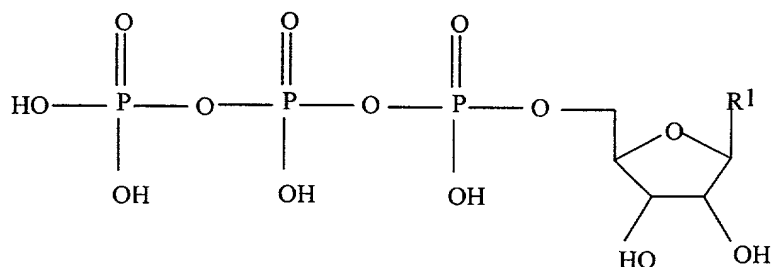
This process is of the same type as the processes described in documents [6] and [7] for forming the morpholino ring.

The nucleotide derivatives of formula (I) may also be prepared from morpholino-nucleosides and the triphosphate group may then be introduced using the

Eckstein protocol, as described by Ludwig et al. in J. Org. Chem. 54, 1989, pages 631-635 [9].

The enzymes which may be used for the enzymatic polymerization may be those described below.

According to the invention, the process for preparing morpholino-nucleotides of formula (I) comprises the reaction of a nucleoside triphosphate of formula:



in which R^1 has the meaning given above, with a periodate, a compound of formula R^2NH_2 in which R^2 has the meaning given above, and sodium borohydride.

The invention also relates to the use of a nucleotide derivative having as precursor a compound of formula (I) for the labelling at the 3' end of nucleic acid (DNA or RNA) fragments by enzymatic incorporation of the nucleotide derivative at the 3' OH end of the nucleic acid fragment.

The invention also relates to the process for manufacturing a 3'-labelled nucleic acid (DNA or RNA) fragment by enzymatic incorporation of the nucleotide derivative mentioned above into the 3' OH end of the nucleic acid fragment.

The enzyme may be the Klenow fragment of the DNA polymerase, and in this case a template is then used to bind the morpholino-nucleoside to the nucleic acid fragment which serves as primer.

The enzyme used may also be a heat-resistant polymerase of a thermophilic bacterium or terminal transferase or reverse transcriptase.

The DNA or RNA fragments thus labelled can be used to block any subsequent ligation and to ensure protection against exonucleases, and also to detect DNA or RNA fragments.

A modified morpholino-nucleotide having as precursor a compound of formula (I) may also be used to modify a nucleic acid (DNA or RNA) fragment by enzymatic incorporation into the 3' end thereof of a modified morpholino-nucleotide having as precursor a compound of formula (I) comprising as R³ a compound chosen from photo-crosslinking agents, for example for crosslinking to DNA or to any support; fatty acids, hydrophobic peptides or antibodies, for example to facilitate the penetration into cells, enzymes or portions of enzymes such as alkaline phosphatases, peroxidases or acetylcholinesterases for the detection, restriction enzymes for cleaving the vicinal DNA, and fluorophores.

As previously, the incorporation of this modified morpholino-nucleotide is carried out enzymatically. The nitrogenous bases, the labels and the enzymes which may be used may be the same as those mentioned above.

According to the invention, the nucleotide derivative, the modified morpholino-nucleotide and the chain terminator used, respectively, for the 3' labelling of nucleic acid fragments, for the modification of nucleic acid fragments or for the sequencing of a nucleic acid, may be the compound (I) in monophosphate form.

Other characteristics and advantages of the invention will emerge more clearly on reading the

description which follows of preparation examples, which are obviously given for illustrative purposes and with no implied limitation, with reference to the attached drawing.

5 **Brief description of the figures**

Figure 1 is a diagram illustrating the results obtained for the sequencing of plasmid DNA with the chain terminator of the invention (solid-line curve) and with the chain terminator of the prior art (dashed-line curve).

Figure 2 is a diagram illustrating the results obtained by testing morpholino A putrescine (MATPP) and morpholino A fluorescein (MATPPF) in sequencing.

Figure 3 is a scheme illustrating the result on polyacrylamide gel of a test for monitoring the elongation of an oligonucleotide A and the incorporation of morpholino A putrescine.

Detailed description of the embodiments

Examples 1 to 4 which follow illustrate the synthesis of morpholino-nucleotides of formula (I).

Example 1: Synthesis of 4-(carboxymethyl)-2-(adenosin-9-yl)-6-(hydroxymethyl)morpholine 6-triphosphate (morpholino A glycine) 1.

This morpholino A glycine 1 corresponds to formula (I) in which R^1 is adenine and R^2 is a $-\text{CH}_2-\text{COOH}$ group.

In this example, all the reactions are carried out at room temperature, with magnetic stirring, in a 50 mL round-bottomed flask.

1.000 g, (1.8 mmol, 1 eq.) of 5'-adenosine triphosphate is dissolved in 10 mL of water and 1 eq. of sodium periodate (388 mg, 1.8 mmol) is then added. The solution is then stirred for 35 minutes.

Glycine (682 mg, 9.1 mmol, 5 eq.) dissolved in 2 mL of water (pH = 9.5-10) is added and the pH of the solution is raised to 9.5-10 with solid potassium carbonate. The solution is stirred for 55 minutes. The reaction mixture turns yellow.

Sodium borohydride (166 mg in total, 4.4 mmol, 2.5 eq.) is added in six equivalent portions, each dissolved in 0.2 mL of water. After adding the first portion, an evolution of gas is noted. The other portions, each dissolved just before addition, are added every hour.

After leaving overnight, the solution is neutralized by adding 1M formic acid to pH 4-5, and is then evaporated.

An analysis by chromatography of reverse-phase polarity on a Merck LiChrocart 125-4 LiChrospher 100 RP-18 column ("endcapped", 5 μ m, 125 \times 4 mm) using a flow rate of 1 mL/min and 25 mM triethylammonium acetate TEAA/methanol MeOH [98/2] as eluent, indicates a yield of 40% (k' = 3.85).

Purification: this is performed by preparative high performance liquid chromatography (HPLC) using a Macherey Nagel Nucleosil 7 C-18 column (7 μ m, 250 \times 21 mm) with a flow rate of 8 mL/min and 25 mM triethylammonium bicarbonate TEAB as eluent.

Characterization:

- ^1H NMR: δ (ppm): 8.47 (s, 1H, H2), 8.37 (s, 1H, H8), 6.26 (dd, 1H, H1'), 4.54 (m, 1H, H4'), 4.28 (m, 1H, H5''), 4.22 (m, 1H, H5'), 3.70 (m, 1H, H2'), 3.68 (s, 2H, CH₂-glycine), 3.41 (m, 1H, H2''), 3.45 (m, 1H, H3'), 3.30 (m, 1H, H3''),

- ^{13}C NMR: δ (ppm): 152.7 (C2), 140.5 (C8), 78.6 (C1'), 74.1 (C4'), 66.4 (C5'), 60.6 (CH₂), 54.5 (C2'), 53.6 (C3')

10 - ^{31}P NMR: δ (ppm): -6.44 (d, 1P, γP), -11.68 (d, 1P, αP), -22.11 (t, 1P, βP)

- Mass spectrometry: $\text{M}-\text{H}^- = 547.04 \text{ g.mol}^{-1}$

15 **Example 2: Synthesis of 4-(carboxymethyl)-2-(thymidin-1-yl)-6-(hydroxymethyl)morpholine 6-triphosphate (morpholino T glycine) 4.**

This compound **4** corresponds to formula (I) with R^1 representing thymine and R^2 representing a $-\text{CH}_2-\text{COOH}$ group.

20 In this example, the morpholino-nucleoside is first prepared and is then converted to a triphosphate.

a) Preparation of the ribothymidine morpholino-nucleoside 2

25 All the reactions are carried out at ambient temperature, with magnetic stirring, in a 250 mL round-bottomed flask.

30 Ribothymidine (3.500 g, 13.5 mmol, 1 eq) is dissolved in 35 mL of water and 1 eq. of sodium periodate (2.900 g, 13.5 mmol) is then added. The solution is then stirred for 45 minutes.

Glycine (5.089 g, 67.8 mmol, 5 eq) in 35 mL of water (pH = 9.5-10) is added and the pH of the solution is raised to 9.5-10 with potassium carbonate. The solution is stirred for 1 hour 45 minutes. The reaction mixture turns yellow.

One sixth of sodium borohydride (1.280 g in total, 33.8 mmol, 2.5 eq) dissolved in 3.5 mL of water is added to the solution. An evolution of gas is noted. The other sixths, each dissolved just before addition, are added every hour.

After leaving overnight, the solution is neutralized by adding 1M formic acid to pH 4-5, and is then evaporated.

An analysis by chromatography of reverse-phase polarity on a Merck LiChrocart 125-4 LiChrospher 100 RP-18 column ("endcapped", 5µm, 125 × 4 mm), with a flow rate of 1 mL/min, using as eluent: 25 mM TEAA/MeOH [99/1], indicates a yield of 32% ($k' = 8.83$).

Purification: this is performed by "flash" chromatography on a column of C-18 silica or reverse-phase polarity (Matrex, Amicon). The eluent is water.

Characterization:

- ^1H NMR: δ (ppm): 7.77 (s, 1H, H6), 5.92 (dd, 1H, H1'), 4.07 (m, 1H, H4'), 3.77 (m, 2H, H5', H5''), 3.22 (s, 2H, CH₂ glycine), 3.13 (dd, 1H, H2''), 2.99 (dd, 1H, H3''), 2.51 (t, 1H, H2'), 2.34 (t, 1H, H3'), 1.98 (s, 3H, CH₃ base).

b) Preparation of the ribothymidine morpholino-nucleoside monophosphate 3

234 μL of phosphorus oxychloride trichloride
5 (2.5 mmol, 1.5 eq.) are added to 342 mg of imidazole
(5.0 mmol, 3 eq) dried in a desiccator and then taken up
in 5 mL of rigorously anhydrous pyridine. The mixture is
stirred for 30 minutes under dry air.

In parallel, 500 mg of the morpholinothymidine
10 (1.7 mmol, 1 eq.) obtained in a) are dried 3 times in
pyridine and then taken up in 5 mL of anhydrous pyridine.

The imidazole/ POCl_3 /pyridine mixture under
argon is added to the morpholinonucleoside solution and
the whole is stirred for 48 hours at ambient temperature.
15 Next, 100 μL of water are added, taking care to cool the
reaction flask in an ice bath. The reaction mixture is
evaporated to dryness and then taken up twice with water
and evaporated in order to remove the pyridine.

An analysis by chromatography of reverse-phase
20 polarity on a Macherey Nagel Nucleosil 5 C-18 column
(7 μm , 120 \times 3 mm), at a flow rate of 1 mL/min, using as
eluent: 25mM TEAA/MeOH [97/3], indicates a yield of 33%
($k' = 0.62$).

25 Purification: this is performed by preparative HPLC on H:
Macherey Nagel Nucleosil 7 C-18 column (7 μm ,
250 \times 21 mm) at a flow rate of 5 mL/min using water as
eluent.

30 Characterization:

- ^1H NMR: δ (ppm): 7.80 (s, 1H, H6), 5.95 (dd,
1H, H1'), 4.19(m, 1H, H4'), 3.94 (t, 2H, H5', H5''), 3.28

(s, 2H, CH₂ glycine), 3.24²⁰ (m, 1H, H2''), 3.10 (m, 1H, H3''), 2.53 (t, 1H, H2'), 2.39 (t, 1H, H3'), 2.00 (s, 3H, CH₃ base)

- ³¹P NMR: δ (ppm) : 1.74 (s)

5

c) Preparation of the ribothymidine morpholino-nucleoside triphosphate 4

1.097 g of carbonyldiimidazole (6.7 mmol, 5 eq.) dissolved in 5 mL of anhydrous dimethylformamide are added to the tributylammonium salt of the thymine morpholinonucleoside monophosphate **3** obtained in b) (511 mg, 1.3 mmol, 1 eq.) dissolved in 3 mL of anhydrous dimethylformamide. The mixture is stirred at ambient temperature for five hours. The excess carbonyl-
diimidazole is destroyed by adding 436 µL of methanol (10.8 mmol, 8 eq.). After 30 minutes, 5 equivalents of tributylammonium pyrophosphate (3.008 g, 6.7 mmol) dissolved in 5 mL of dimethylformamide are added. The mixture is stirred for 2 days and the reaction mixture is then filtered and evaporated to dryness.

An analysis by chromatography of reverse-phase polarity is carried out on an SFCC PVDI 31 column (5 µm, 100 × 4.6 mm), at a flow rate of 1 mL/min, using as eluent a gradient of ammonium formate (AF), under the following conditions:

t (min)	25 mM AF (%)	0.9 M AF (%)
0	100	0
10	100	0
40	0	100
41	0	100
43	100	0

This indicates a yield of 27% ($k' = 13.84$).

Purification: this is performed by "flash" chromatography on a column of ion-exchange phase (DEAE Sepharose Fast Flow, Pharmacia Biotech). The eluent is a gradient of TEAB (from 25 mM to 0.9 M).

Characterization :

- ^1H NMR: δ (ppm) : 7.74 (s, 1H, H6), 5.92 (dd, 1H, H1'), 4.25 (m, 1H, H4'), 4.15 (m, 2H, H5', H5''), 3.81 (s, 2H, CH₂ glycine), 3.54 (d, 1H, H2''), 3.10 (t, 1H, H3''), 2.56 (t, 1H, H2'), 2.45 (t, 1H, H3'), 1.95 (s, 3H, CH₃ base)

- ^{31}P NMR: δ (ppm): -10.03 (d, 1P, γP), -10.88 (d, 1P, αP), -22.65 (t, 1P, βP)

- Mass spectrometry: $\text{M-H}^- = 540,41 \text{ g.mol}^{-1}$

Example 3: Synthesis of 4-(carboxymethyl-2-(guanine-9-yl)-6-(hydroxymethyl)morpholine 6-triphosphate (morpholino G glycine) 5.

This morpholino G glycine 5 corresponds to formula (I) with $\text{R}^1 = \text{guanine}$ and $\text{R}^2 = -\text{CH}_2\text{COOH}$.

Guanosine 5'-triphosphate (50 mg, 0.08 mmol, 1 eq.) is dissolved in 2 mL of water and 1 eq. of sodium periodate (18 mg, 0.08 mmol) is then added. The solution is then stirred for 35 minutes. Glycine (31 mg, 0.42 mmol, 5 eq.) dissolved in 2 mL (pH = 9.5-10) [lacuna] is added and the pH of the solution is raised to 9.5-10 with solid potassium carbonate (monitored with pH paper). The solution is stirred for 45 minutes. Sodium borohydride (8 mg in total, 0.21 mmol, 2.5 eq.) is added

in six equivalent portions, each dissolved in 0.1 mL of water. The other fractions, each dissolved just before addition, are added every hour. After leaving overnight, the solution is neutralized by adding 1M formic acid to pH 4-5 and is then evaporated.

An analysis by chromatography of reverse-phase polarity (System E) on an SFCC PVDI 31 column (5 μ m, 100 \times 4.6 mm), with a flow rate: 1 mL/min, using as eluent a gradient of ammonium formate, under the following conditions:

t (min)	25 mM AF (%)	1 M AF (%)
0	100	0
3	100	0
10	0	100
15	0	100
17	100	0

This analysis gives a yield of 39% (k' = 5.5).

Compound **5** is purified by preparative HPLC using System F: Vydac Sax-Protein column (8 μ m, 100 \times 4.6 mm). Flow rate: 10 mL/min. Eluent: gradient of ammonium formate, under the following conditions:

t (min)	25 mM AF (%)	1 M AF (%)
0	100	0
3	100	0
10	0	100
15	0	100
17	100	0

14 mg of compound **5** are obtained, i.e. a yield of 26.1%.

Characterization:

- ^1H NMR (Brüker AM 400): δ (ppm): 8.07 (s, 1H, H8); 6.06 (dd, 1H1, H1') 4.51 (m, 1H, H4'); 4.22 (m, 2H, H5', H5''); 3.71 (m, 1H, H2''); 3.67 (s, 2H, -CH₂ glycine); 3.46 (m, 1H, H3''); 3.38 (m, 1H, H2'); 2.95 (m, 1H, H3').

- ^{13}C NMR (Brüker AM 400): δ (ppm): 173.50 (-COOH); 158.91 (C6); 153.98 (C2); 151.07 (C4); 137.39 (C8); 115.94 (C5); 77.87 (C 1'); 73.62 (C4'); 65, 61 (C5'); 59.98 (-CH₂-); 53.28 (C2'); 51.88 (C3').

- ^{31}P NMR (U 400 Varian): δ (ppm): -7.14 (d, 1P, γP); 8.68 (d, 1P, αP); -20,28 (t, 1P, βP).

- Mass spectrometry (LCQ machine in positive mode): $\text{M}+\text{H}^+ = 564.9 \text{ g.mol}^{-1}$.
 - UV spectrum: $\lambda_{\text{max}} = 256 \text{ nm}$.
 - Capillary electrophoresis:
 $\mu_{\text{ep}} = -4.28 \times 10^{-4} \text{ cm}^2.\text{V}^{-1}.\text{s}^{-1}$.

Example 4: Synthesis of 4-(carboxymethyl)-2-(cytosin-1-yl)-6-(hydroxymethyl)morpholine 6-triphosphate (morpholino C glycine) 6

Compound **6** corresponds to formula (I) with $\text{R}^1 = \text{cytosine}$ and $\text{R}^2 = -\text{CH}_2-\text{COOH}$.

All the reactions are carried out at ambient temperature, with magnetic stirring, in a 20 mL round-bottomed flask.

The reaction is the same as for compound **5**, starting with cytosine 5'-triphosphate (50.0 mg, 0.09 mmol, 1 eq.), sodium periodate (21 mg, 0.09 mmol,

1 eq.), glycine (36 mg, 0.48 mmol, 5 eq.) dissolved in 2 mL of water (pH = 9.5-10) and sodium borohydride (9 mg in total, 0.23 mmol, 2.5 eq.), added in six equivalent portions, each dissolved in 0.05 mL of water.

5 An analysis by chromatography on an ion-exchange phase column (System E), as in Example 3, indicates a capacity factor $k' = 4.08$.

The product is purified by semi-preparative HPLC using System F as in Example 3.

10 17 mg of product are isolated, which corresponds to a yield of 24.3%.

Characterization:

15 - ^1H NMR (Brüker AM 400): δ (ppm): 7.93 (d, 1H, H6); 6.25 (dd, 1H, H1'); 6.20 (d, 1H, H5); 4.51 (m, 1H, H4'); 4.27 (m, 2H, H5', H5''); 3.85 (m, 4H, H2'' + H3'' + $-\text{CH}_2$ glycine); 3.33 (t, 1H, H2'); 3.22 (t, 1H, H3').

20 - ^{13}C NMR (Brüker AM 400): δ (ppm): 173.05 ($-\text{COOH}$); 165.13 (C4); 154.23 (C2); 140.93 (C6); 95.48 (C5); 80.42 (C1'); 78.44 (C4'); 69.37 (C5'); 64.57 ($-\text{CH}_2-$); 54.66 (C2'); 53.67 (C3').

- ^{31}P NMR (Brüker WM 250): δ (ppm): -7.99 (d, 1P, γP); -10, 10 (d, 1P, αP); -21.28 (t, 1P, βP).

25 - Mass spectrometry
(VG ZAB-2-EQ machine, negative mode):
 $\text{M} - \text{H}^- = 521.9 \text{ g.mol}^{-1}$.

- UV spectrum: $\lambda_{\text{max}} = 270 \text{ nm}$

- Capillary electrophoresis :

$$\mu_{\text{ep}} = -4.28 \times 10^{-4} \text{ cm}^2.\text{V}^{-1}.\text{s}^{-1}.$$

Example 5: Synthesis of 4-(aminobutyl)-2-(adenosin-9-yl)-6-(hydroxymethyl)morpholine 6-triphosphate (morpholino A putrescine) 7.

This morpholino A putrescine 7 corresponds to
5 formula (I) with R^1 representing adenine and R^2
representing a $-(CH_2)_4-NH_2$ group.

All the reactions are carried out at ambient
temperature, with magnetic stirring, in a 100 mL flask.

Adenosine 5'-triphosphate (500 mg, 0.9 mmol,
10 1 eq.) is dissolved in 10 mL of water and 1 eq. of sodium
periodate (194 mg, 0.9 mmol) is then added. The solution
is then stirred for 45 minutes.

Putrescine (456 μ L, 4.5 mmol, 5 eq.) is added.
The solution is stirred for 45 minutes. The reaction
15 mixture turns yellow.

One sixth of sodium borohydride (86 mg in
total, 2.3 mmol, 2.5 eq.) dissolved in 0.1 mL of water is
added to the solution. An evolution of gas is noted. The
other sixths, each dissolved just before addition, are
20 added every hour.

After leaving overnight, the solution is
neutralized by adding 1M formic acid to pH 4-5 and is
then evaporated.

An analysis by chromatography of reverse-phase
25 polarity is carried out on a Merck LiChrocart 125-4
LiChrospher 100 RP-18 column ("endcapped", 5 μ m,
125 x 4mm) with a flow rate of 1 mL/min, using as eluent
a 25 mM TEAB/MeOH gradient, under the following
conditions:

30

	26	
t (min)	TEAB	MeOH
	(%)	(%)
0	97	3
2	97	3
10	90	10
15	90	10
17	97	3

This analysis indicates a yield of 67% ($k' = 3.81$).

Product **7** is purified by semi-preparative HPLC on a Phenomenex Ultremex 5-C18 column (250 × 10 mm) with a flow rate of 4 mL/min, and using as eluent a 25 mM TEAB/MeOH gradient, under the following conditions:

t (min)	TEAB	MeOH
	(%)	(%)
0	95	5
3	95	5
8	90	10
10	95	5

10 Characterization:

- ^1H NMR: δ (ppm): 8.44 (s, 1H, H2), 8.33 (s, 1H, H8), 6.06 (dd, 1H, H1'), 4.35 (m, 1H, H4'), 4.22 (m, 2H, H5', H5''), 3.39 (d, 1H, H2'), 3.22 (t, 1H, H3''), 3.14 (s, 2H, CH₂ putrescine), 2.92 (t, 1H, H2'), 2.74 (s, 2H, CH₂ putrescine), 2.54 (t, 1H, H3'), 1.78 (s, 4H, (CH₂)₂ putrescine).

- ^{31}P NMR: δ (ppm): -8.45 (dd, 1P, γP), -13.25 (dd, 1P, αP), -24.20 (t, 1P, βP)

- Mass spectrometry: $M + \text{H}^+ = 561.92 \text{ g.mol}^{-1}$

5 **Example 6: Synthesis of 4-(aminobutyl)-2-(thymidin-1-yl)-6-(hydroxymethyl)morpholine 6-triphosphate (morpholino T putrescine) 9.**

Compound 9 corresponds to formula (I) with $\text{R}^1 = \text{thymine}$ and $\text{R}^2 = -(\text{CH}_2)_4\text{-NH}_2$.

10 a) Preparation of 4-(aminobutyl)-2-(thymidin-1-yl)-6-(hydroxymethyl)morpholine-6-hydroxyl 8.

All the reactions are carried out at ambient temperature, with magnetic stirring, in a 250 mL round-bottomed flask.

15 Ribothymidine (2.000 g, 7.74 mmol, 1 eq.) is dissolved in 30 mL of water and 1 eq. of (1.656 g, 7.75 mmol) of sodium periodate is then added. The solution is then stirred for 70 minutes. Putrescine (3.9 mL, 38.75 mmol, 5 eq.) is added. The solution is stirred for 50 minutes. The reaction mixture turns yellow.

20 One sixth of sodium borohydride (735 mg in total, 19.42 mmol, 2.5 eq.) dissolved in 0.25 mL of water is added to the solution. An evolution of gas is noted. The other sixths, each dissolved just before addition to 0.25 mL of water, are added every hour.

25 After leaving overnight, the solution is neutralized by adding 1M formic acid to pH 4-5 and is then evaporated. An analysis by chromatography of reverse-phase polarity is carried out using System G: Merck LiChrocart 125-4 LiChrospher 100 RP-18 column

30

("endcapped", 5 μ m, 125 \times 4 mm). Flow rate: 1 mL/min. Eluent: 25 mM TEAB/CH₃CN gradient, under the following conditions:

t (min)	25 mM TEAB (%)	CH ₃ CN (%)
0	100	0
4	100	0
15	85	15
18	100	0

5 This indicates a 76% yield ($k' = 5.7$).

The product is purified by preparative HPLC using System H: Macherey Nagel Nucleosil 7 C-18 column (7 μ m, 250 \times 21 mm). Flow rate: 10 mL/min. Eluent: 25 mM TEAB/CH₃CN [85/15].

10 1.56 g of compound **8** are obtained, i.e. a 64.6% yield.

Characterization:

15 - ¹H NMR 1H (Brüker AC 200): δ (ppm): 7.69 (s, 1H, H6); 5.88 (dd, 1H, H1'); 4.01 (m, 1H, H4'); 3.80 (m, 1H, H5', H5''); 3.08 (m, 4H, H2'', H3'', 2Ha); 2.63 (m, 2H, 2 Hd); 2.33 (t, 1H, H2'); 2.22 (t, 1H, H3'); 1.98 (m, 3H, -CH3); 1.74 (m, 4H, 2 Hb, 2 Hc).

20 - ¹³C NMR (Brüker AC 200): δ (ppm): 171.16 (C2); 154.58 (C4); 135.93 (C6); 110.46 (C5); 78.62 (C1'); 75.04 (C4'); 61.10 (C5'); 55.82 (C3'); 53.49 (C2'); 51.30 (Ca); 38.39 (Cd); 24.50 (Cc); 21.31 (Cb); 11.10 (-CH₃).

- UV spectrum: $\lambda_{\text{max}} = 266$ nm.

b) Preparation of 4-(aminobutyl)-2-(thymidin-1-yl)-6-(hydroxymethyl)morpholine 6-triphosphate 9.

Morpholinothymidine/putrescine **8** (249 mg, 0.80 mol, 1 eq.) is dried using a vane pump for 1 hour. 256 mg of Proton-sponge® (1.19 mmol, 1.5 eq.) are then added and 2 mL of anhydrous trimethyl phosphate are added; The medium is placed in an ice bath, with stirring, and 109 µL of phosphorus oxychloride are then added (2.24 mmol in total, 2.8 eq.). After 2 h 30 min, a further 50 mL of phosphorus oxychloride are added, and this operation is repeated 12 h later. Next, 8 mL of a 0.5M solution of pyrophosphate in the form of the tributylammonium salt (4.0 mmol, 5 eq.), in anhydrous DMF are added. The mixture is stirred at 0°C for one minute and the medium is then dried on a rotavapor and vane pump.

An analysis by chromatography of reverse-phase polarity using System I: Vydac Sax-Protein column (8 µm, 100 × 4.6 mm) with a flow rate: 10 mL/min, using as eluent a gradient of ammonium formate, under the following conditions:

t (min)	25 mM AF (%)	1M AF (%)
0	100	0
1	100	0
15	70	30
17	100	0

This indicates a capacity factor $k' = 3.2$.

The product is purified by preparative HPLC

using System I described above.

48 mg of **9** are obtained, i.e. a 13.2% yield.

Characterization:

- 5 - ^1H NMR (Brüker AM 400): δ (ppm): 7.83 (s, 1H, H6); 6.31 (dd, 1H, H1'); 4.68 (m, 1H, H4'); 4.39 (m, 1H, H5', H5''); 4.01 (d, 1H, H2''); 3.93 (d, 1H, H3''); 3.58 (m, 2H, 2 Ha); 3.51 (t, 1H, H2'); 3.41 (m, 1H, H3'); 3.28 (m, 2H, 2 Hd); 2.10 (s, 5H, $-\text{CH}_3 + 2 \text{ Hb}$); 2.00 (m, 2H, 2Hc).
- 10 - ^{13}C NMR (Brüker AM 400): δ (ppm): 166.36 (C2); 151.03 (C4); 136.73 (C6); 112.42 (C5); 77.33 (C1'); 72.46 (C4'); 65.10 (C5'); 57.04 (C3); 51.71 (C2'); 51.13 (Ca); 98.91 (Cd); 23.85 (Cc); 20.50 (Cb); 11.62 ($-\text{CH}_3$).
- ^{31}P NMR (U 400 Varian): δ (ppm): -8,19 (s, 2P, γP , αP); -18,99 (t, 1P, βP).
- 15 - Mass spectrometry (LCQ machine in negative mode): $\text{M} - \text{H}^- = 551.3 \text{ g.mol}^{-1}$.
- UV spectrum: $\lambda_{\text{max}} = 262 \text{ nm}$.
- Capillary electrophoresis:
- 20 $\mu\text{ep} = -4.69 \times 10^{-4} \text{ cm}^2.\text{V}^{-1}.\text{s}^{-1}$.

Example 7: Synthesis of 4-(aminobutyl)-2-(guanosin-9-yl)-6-(hydroxymethylmorpholine 6-triphosphate (morpholino G putrescine) 10.

- 25 Compound **10** corresponds to formula (I) with $\text{R}^1 = \text{guanine}$ and $\text{R}^2 = -(\text{CH}_2)_4-\text{NH}_2$.

All the reactions are carried out at ambient temperature, with magnetic stirring, in a 50 mL round-bottomed flask.

- 30 Guanosine 5'-triphosphate (50 mg, 0.17 mmol,

1 eq.) is dissolved in 5 mL of water and 1 eq. of sodium periodate (37 mg, 0.17 mmol, 1 eq.) is then added. The solution is then stirred for 30 minutes.

Putrescine (85 μ L, 0.84 mmol, 5 eq.) is added and the pH of the solution is measured, and is equal to 10. If a lower value had been found, potassium carbonate would have been added to obtain this value. The solution is stirred for 45 minutes.

Sodium borohydride (8.7 mg in total, 0.45 mmol, 2.5 eq.) is added in six equivalent portions, each dissolved in 0.1 mL of water. The other fractions, each dissolved just before addition, are added every hour.

After leaving overnight, the solution is neutralized by adding 1M formic acid to pH 4-5 and is then evaporated.

Compound **10** is purified by precipitation from methanol followed by passage through 5 mL of Dowex resin in Na⁺ form.

68 mg of compound **10** are obtained, i.e. a yield of 62.2%.

Characterization:

- ¹H NMR (Brüker AM 400): δ (ppm): 8.29 (s, 1H, H8); 6.31 (dd, 1H, H1'); 4.74 (m, 1H, H4'); 4.37 (m, 2H, H5', H5''); 3.99 (m, 1H, H2''); 3.96 (m, 1H, H3''); 3.79 (t, 1H, H2'); 3.47 (m, 2H, 2 Hb); 3.39 (t, 1H, H3'); 3.19 (m, 2H, 2 Hc); 2.06 (m, 2H, 2 Ha); 1.91 (m, 2H, 2 Hd).

- ¹³C NMR (Brüker AM 400): δ (ppm): 151.11 (C6); 154.11 (C2); 149.91 (C4); 136.95 (C8); 113.46 (C5); 76.99 (C1'); 72.58 (C4'); 65.25 (C5'); 56.95 (Ca); 51.81 (C2'); 50.52 (C3'); 30.04 (Cd); 23.76 (Cc); 20.36 (Cb).

- ^{31}P NMR (U 400 Varian): δ (ppm): -8.28 (d, 1P, γP); -8.97 (d, 1P, αP); -20.45 (t, 1P, βP).

- Mass spectrometry (LCQ machine in negative mode): $M - \text{H}^- = 576.9 \text{ g.mol}^{-1}$.
- UV spectrum: $\lambda_{\text{max}} = 252 \text{ nm}$.
- Capillary electrophoresis:
 $\mu_{\text{ep}} = -3.41 \times 10^{-4} \text{ cm}^2.\text{V}^{-1}.\text{s}^{-1}$.

Example 8: Synthesis of 4-(aminobutyl)-2-(cytosin-1-yl)-6-(hydroxymethylmorpholine 6-triphosphate (morpholino C putrescine) 11

The entire reaction is carried out at ambient temperature, with magnetic stirring, in a 50 mL round-bottomed flask.

The reaction is the same as for compound 7, starting with cytosine 5'-triphosphate (50 mg, 0.09 mmol, 1 eq.), sodium periodate (20 mg, 0.09 mmol, 1 eq.), putrescine (47 μL , 0.47 mmol, 5 eq) and sodium borohydride (9.1 mg in total, 0.24 mmol, 2.5 eq.) added in six equivalent portions, each dissolved in 0.1 mL of water.

An analysis by chromatography of reverse-phase polarity (System O): Merck Lichrocart 125-4 LiChrospher 100RP-18 column ("endcapped", 5 μm , 125 \times 4 mm). Flow rate: 1 mL/min. Eluent: 25 mM TEAB/MeOH gradient, under the following conditions:

t(min)	25 mM TEAB (%)	MeOH (%)
0	97	3
2	97	3
10	90	10
15	90	10
17	97	3

5 ... **indicates** a capacity factor $k' = 4.18$.

Compound **11** is purified by precipitation from methanol and then passage through 5 mL of Dowex resin in Na^+ form.

10 47 mg of **11** are obtained, which corresponds to to a yield of 85.4%.

Characterization:

15 - ^1H NMR (Brüker AM 400): δ (ppm): 7.78 (d, 1H, H6); 6.17 (d, 1H, H5); 5.96 (dd, 1H, H1'); 4.22 (m, 1H, H4'); 3.91 (m, 2H, H5', H5''); 3.28 (m, 1H, H2''); 3.20 (m, 1H, H3''); 3.16 (m, 2H, 2 Ha); 2.80 (m, 2H, 2 Hd); 2.44 (m, 1H, H2'); 2.32 (m, 1H, H3'); 1.79 (m, 4H, 2 Hb + 2 Hc)

20 - ^{13}C NMR (Brüker AM 400): δ (ppm): 166.056 (C4); 157.28 (C2); 142.43 (C6); 96.88 (C5); 80.57 (C1'); 75.13 (C4'); 66.48 (C5'); 57.11 (Ca); 55.30 (C2'); 52.45 (C3'); 30.66 (Cd); 25.29 (Cc); 22.70 (Cb).

25 - ^{31}P NMR (Brüker WM 250): δ (ppm): -5.42 (d, 1P, γP); -10.06 (d, 1P, αP); -20.82 (m, 1P, βP).

- Mass spectrometry (LCQ machine in negative mode): $M - H^- = 536.0 \text{ g.mol}^{-1}$.
- UV spectrum: $\lambda_{\text{max}} = 268 \text{ nm}$
- Capillary electrophoresis:
 $\mu_{\text{ep}} = -2.99 \times 10^{-4} \text{ cm}^2.\text{V}^{-1}.\text{s}^{-1}$.

Example 9: Synthesis of 4-[5((2-aminobutyl)-thioureidyl)fluorescein)]-2-(adenosin-9-yl)-6-(hydroxymethyl)morpholine 6-triphosphate (morpholino A putrescine-fluorescein) 12.

This compound **12** corresponds to formula (I) with R^1 representing adenine and R^2 representing $(\text{CH}_2)_4\text{NHR}^3$ in which R^3 is a group derived from fluorescein.

All the reactions are carried out at ambient temperature, with magnetic stirring, in a 100 mL round-bottomed flask.

184.9 mg (0.5 mmol, 1.5 eq.) of fluorescein isothiocyanate are added gradually in three portions to 200 mg (0.3 mmol, 1 eq.) of morpholino A putrescine **7** of Example **5**, in a water/pyridine mixture (1/1). The medium is stirred for 48 hours and then evaporated to dryness.

An analysis by chromatography of reverse-phase polarity on a Merck LiChrocart 125-4 LiChrospher 100 RP-18 column ("endcapped", 5 μm , 125 \times 4 mm), with a flow rate of 1 mL/min using as eluent: 25 mM TEAA/MeOH [97/3], indicates a yield of about 48% ($k' = 7.51$).

Purification: this is performed by "flash" chromatography on a column of C-18 silica of reverse-phase polarity (Econosil prep 90, Alltech, France). The eluent is a water/MeOH gradient.

Characterization:

- ^1H NMR: δ (ppm): 8.57 (s, 1H, H2), 8.31 (s, 1H, H8), 8.20-6.65 (9H, fluorescein), 5.79 (dd, 1H, H1'), 4.25 (m, 1H, H4'), 4.11 (m, 2H, H5', H5''), 3.60 (s, 2H, CH₂ putrescine), 3.12 (d, 1H, H3''), 2.93 (d, 1H, H2''), 2.81 (m, 1H, H2'), 2.59 (m, 2H, CH₂ putrescine), 2.50 (dd, 1H, H3'), 1.79 (s, 2H, CH₂ putrescine), 1.62 (m, 2H, CH₂ putrescine)

- ^{31}P NMR: δ (ppm): -8.45 (dd, 1P, γP), -13.25 (dd, 1P, αP), -24.20 (t, 1P, βP)

Mass spectrometry: $\text{M-H}^- = 949.2 \text{ g.mol}^{-1}$

Example 10: Synthesis of 4-[5(((2-aminobutyl)-thioureidyl)fluorescein)-1-2-(thymidin-1-yl)-6-(hydroxymethyl)morpholine 6-triphosphate (morpholino T putrescine fluorescein) 13

All the reactions are carried out at ambient temperature, with magnetic stirring, in a 25 mL round-bottomed flask.

31 mg (0.08 mmol, 1.5 eq.) of fluorescein isothiocyanate are added in three portions to 30 mg (0.05 mmol, 1 eq.) of compound **9** dissolved in 2 mL of a water/pyridine mixture (1/1). The medium is stirred for 48 hours and then evaporated to dryness.

Compound **13** is purified by semi-preparative high performance liquid chromatography, on a column of reverse-phase polarity (System L): Macherey Nagel Nucleosil 7 C-18 column (7 μm , 250 \times 21 mm). Flow rate: 10 mL/min. Eluent: 25 mM TEAB/CH₃CN, under the following conditions:

t (min)	25 mM TEAB (%)	CH ₃ CN (%)
0	100	0
4	100	0
15	73	27
18	100	0

Characterization:

- Mass spectrometry (LCQ machine in positive mode):
5 M-H⁺ = 942.1 g.mol⁻¹.

- UV spectrum: λ_{max} = 488 nm.

- Capillary electrophoresis:

$$\mu_{\text{cp}} = -4.23 \times 10^{-4} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}.$$

10

Example 11: Synthesis of 4-[5(((2-aminobutyl)-thioureidyl)fluorescein)]-2-(guanosin-9-yl)-6-(hydroxymethyl)morpholine 6-triphosphate (morpholino G putrescine fluoresceine) 14.

15

All the reactions are carried out at ambient temperature, with magnetic stirring, in a 25 mL round-bottomed flask.

30 mg (0.08 mmol, 1.5 eq.) of fluorescein isothiocyanate are added gradually in three portions to
20 30 mg (0.05 mmol, 1 eq.) of compound **10** dissolved in 2 mL of a water/pyridine mixture (1/1). The medium is stirred for 48 hours and then evaporated to dryness.

An analysis by chromatography of reverse-phase polarity (System M): Merck-LiChrochart 125-4 LiChrospher
25 100 RP-18 column ("endcapped", 5 μm , 125 \times 4 mm). Flow rate: 1 mL/min. Eluent: 25 mM TEAB/CH₃CN gradient, under

the following conditions:

t(min)	25 mM TEAB (%)	CH ₃ CN (%)
0	100	0
4	100	0
15	73	27
18	100	0

indicates a yield of about 24% ($k' = 4.62$).

Compound **14** is purified by semi-preparative high performance liquid chromatography, on a column of reverse polarity, using System L of Example 10.

14.5 mg of compound **14** are obtained, i.e. a yield of 30.0%.

10 Characterization:

- ¹H NMR (Brüker AM 400): δ (ppm): 7.87 (s, 1H, H8); 7.70-6.63 (9H, fluorescein); 5.60 (dd, 1H, H1'); 4.18 (m, 1H, H4'); 4.12 (m, 2H, H5', H5''); 3.82 (m, 1H, Ha); 3.61 (m, 1H, Ha); 3.08 (d, 1H, H3''); 2.95 (d, 1H, H2''); 2.82 (m, 1H, H2'); 2.71 (m, 1H, Hd); 2.55 (m, 1H, Hd); 2.39 (t, 1H, H3'); 1.77 (m, 2H, 2 Hb); 1.62 (m, 2H, 2 Hc).

- ¹³C NMR (Brüker AM 400): δ (ppm): 180.58 (several fluorescein C); 158.37 (several fluorescein C); 136.98 (C6); 131.06 (C2); 126.7 (C4); 122.85 (several fluorescein C); 112.03 (C8); 103.80 (several fluorescein C); 78.91 (C1'); 74.83 (C4'); 65.96 (C5'); 57.27 (Ca); 53.79 (C2'); 52.56 (C3'); 48.87 (Cd); 25.70 (Cc); 22.75 (Cb).

- ³¹P NMR (U 400 Varian): δ (ppm): -4.93 (dd, 1P, γ P); -9.82 (d, 1P, α P); -19.94 (t, 1P, β P).

- Mass spectrometry (LCQ machine in negative mode): $M - H^- = 985.3 \text{ g.mol}^{-1}$.

- UV spectrum: $\lambda_{\text{max}} = 494 \text{ nm}$

5

- Capillary electrophoresis:

$$\mu_{\text{ep}} = -3.83 \times 10^{-4} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}.$$

Example 12: Synthesis of 4-[5(((2-aminobutyl)-thioureidyl)fluorescein)]-2-(cytosin-1-yl)-6-(hydroxymethyl)morpholino 6-triphosphate (morpholino C putrescine-fluorescein) 15.

10

All the reactions are carried out at ambient temperature, with magnetic stirring, in a 10 mL round-bottomed flask.

15

36 mg (0.09 mmol, 1.5 eq.) of fluorescein isothiocyanate are added in three portions to 30 mg (0.05 mmol, 1 eq.) of compound **11**, dissolved in 2 mL of a water/pyridine mixture (1/1). The medium is stirred for 48 hours and then evaporated to dryness.

20

An analysis by chromatography of reverse-phase polarity (System M described in Example 11) indicates a capacity factor $k' = 4.7$.

Compound **15** is purified by semi-preparative high performance liquid chromatography, on a column of reverse-phase polarity (System L of Example 10).

25

22.7 mg of compound **15** are obtained, i.e. a yield of 44.3%.

Characterization:

30

- ^1H NMR (Brüker AM 400): δ (ppm): 7.99 (s, 1H, H6); 7.87-6.69 (9H, fluorescein); 5.78 (d, 2H, H5 + H1'); 4.14 (m, 1H, H4'); 3.77 (m, 2H, H5', H5''); 3.36

(m, 2H, 2 Ha); 3.32 (m, 1H, H2''); 3.03 (m, 1H, H3''); 2.81 (m, 1H, H2'); 2.69 (m, 2H, 2 Hd, 1,79); 2.30 (m, 1H, H3'); 1.79 (m, 2H, 2 Hb); 1.68 (m, 2H, 2 Hc)

- ^{13}C NMR (Brüker AM 400): δ (ppm): 175.06
 5 (several fluorescein C); 157.62 (C2); 141.39 (several fluorescein C); 131.56 (C6); 121.06 (several fluorescein C); 114.60 (several fluorescein C); 103.30 (several fluorescein C); 96.53 (C5); 79.10 (C1'); 73.67 (C4'); 65.42 (C5'); 58.89 (Ca); 57.19 (C2'); 51.78 (C3'); 46.61
 10 (Cd); 25.48 (Cc); 21.38 (Cb)

- ^{31}P NMR (U 400 Varian): δ (ppm): -2.97 (d, 1P, γP); -7.54 (d, 1P, αP); -18,56 (m, 1P, βP).

- Mass spectrometry (LCQ machine in negative
 15 mode): $\text{M} - \text{H}^- = 925.2 \text{ g.mol}^{-1}$.

- UV spectrum: $\lambda_{\text{max}} = 491 \text{ nm}$.

- Capillary electrophoresis:

$$\mu_{\text{ep}} = -4.26 \times 10 \text{ cm}^2.\text{V}^{-1}.\text{s}^{-1}.$$

20 **Example 13: Use of morpholino T glycine for the analysis of a DNA sequence**

The morpholino T glycine **4** of Example 2 is tested in sequence reaction with fluorescent primers (Applied Biosystems, Perkin-Elmer, Foster City, CA, USA)
 25 on a standard template which is a Bluescript plasmid DNA (Stratagene, La Jolla, CA, USA). The enzyme used is a Taq polymerase (Perkin-Elmer), which is used in its buffer (TACS buffer, Perkin-Elmer).

Two reactions are carried out with morpholino
 30 T glycine at 200 and 500 μM (Table 4), and also two

control reactions (Table 5) with dideoxynucleotide T (Boehringer).

The reaction medium, of a total volume of 10 μ L, contains 125 ng of template, 1.25 pmol of fluorescent primer and the other constituents given in Tables 4 and 5. The medium is subjected to heat cycles in order to produce in number molecules of newly formed DNA strands. An amplification on an Perkin-Elmer 9700 machine is performed, according to the following sequences:
3 min., 95°C; 15 cycles (15 sec., 95°C; 15 sec., 55°C; 1 min., 70°C); 15 cycles (15 sec., 95°C; 1 min., 70°C). The amplification product is purified on a Sephadex G50 column.

The migration of the amplification product obtained in the column eluate is performed in denaturing gel (7M urea) of acrylamide of Long Ranger type (6%), in 1X TBE, on an Applied Biosystems 377 machine. The electrophoresis is carried out for 12 hours under 1500 V.

The preparation of the stock solution of nucleotides representing in this case a mixture of the four natural nucleoside triphosphates, depleted in thymidine triphosphate (known as dTTP mix) is carried out in the following way.

2 μ L of a 1.25 mM solution of dTTP (Promega) are mixed with 2 μ L of 5 mM dATP (Promega), 2 μ L of 5 mM dCTP (Promega) and 2 μ L of 5 mM dGTP (Promega).

Table 4

	200 μM Morpholino T glycine	500 μM Morpholino T glycine
TACS buffer (x5)	2 μ L	2 μ L
Z1M13 Primer (JOE)	1 μ L	1 μ L
DTTP mix	1 μ L	1 μ L
2 mM Morpholino T glycine	1 μ L	2.5 μ L
Taq (3U/ μ L)	1 μ L	1 μ L
Template	1 μ L	1 μ L
H ₂ O	3 μ L	1.5 μ L

Table 5

	ddTTP 250 μM	ddTTP 300 μM
TACS buffer (x5)	2 μ L	2 μ L
Z1M13 Primer (ROX)	1 μ L	1 μ L
DTTP mix	1 μ L	1 μ L
2.5 mM DdTTP	1 μ L	2.5 μ L
Taq (3U/ μ L)	1 μ L	1 μ L
Template	1 μ L	1 μ L
H ₂ O	3 μ L	1.5 μ L

5

The products of the sequencing reactions are detected by fluorescence. The results obtained are represented in the attached figure which illustrates the detection of the products in the sequencing gel analysed by the Perkin-Elmer Analysis software, version 3.0.

For each test, the primers are identifiable by their fluorescence properties, the ROX label (red) for the control reaction 250 μ m dideoxythymidine triphosphate (dashed-curve) and the label JOE (green) for the reaction concerning the 200 μ m morpholino T glycine (solid-line curve).

As shown in the figure, the results of these tests are entirely conclusive since the morpholino T glycine is correctly incorporated in a base-specific manner by the Taq polymerase, and acts correctly as a chain terminator.

The other three morpholino-nucleotides **1**, **5** and **6** may be used in the same manner to determine the positions of the four DNA bases in the fragment to be analysed.

Example 14: Testing morpholino A putrescine and morpholino A fluorescein in sequencing

Morpholino A putrescine (MATPP) **7** and morpholino A fluorescein (MATPPF) **12** are tested in sequencing reaction with fluorescent primers (Applied Biosystems, Perkin-Elmer, Foster City, CA, USA) on a standard template which is a Bluescript plasmid DNA (Stratagene, La Jolla, CA, USA). The enzyme used is a Taq polymerase (Perkin-Elmer) which is used in its buffer (Thermo Sequenase buffer, Amersham Life Science).

Three sequencing reactions are carried out with MATPP at 100, 200 and 400 μM and four sequencing reactions are carried out with MATPPF at 200, 500, 1 000 and 5 000 μM , along with control reactions with the dideoxynucleotide ddATP at a concentration of 250 μM (Boehringer).

The reaction medium, of a total volume of 10 μL , contains 125 ng of template, 1.25 pmol of fluorescent primer and the other constituents as described in the tables.

The medium is subjected to heating cycles in order to produce in number molecules of newly formed DNA strands. An amplification on a Perkin-Elmer 9700 machine

(Gene Amp[®], PCR System 9700) is carried out, according to the following sequences:

MATPP 7 3 min, 95°C; 30 cycles (15 sec., 95°C; 15 sec., 55°C; 1 min, 70°C)

MATPPF 12 3 min, 95°C; 30 cycles (15 sec., 95°C; 15 sec., 55°C; 4 min, 60°C)

- 5 The amplification products are purified on a Sephadex G50 column. The products of each sequencing reaction are mixed with the products of a control reaction and analysed by electrophoresis.

- 10 The migration of the mixture obtained is carried out in denaturing gel (7M urea) of acrylamide of Long Ranger type (6%), in 1X TBE, on an Applied Biosystems 377 machine (ABI Prism DNA Sequencer, Perkin-Elmer). The electrophoresis is carried out for 7 hours under 1680 V, 50 mA.

- 15 **Preparation of the stock solution of nucleotides: dATP mix for 16 reactions**

[lacuna] representing in this case a mixture of the four natural nucleotide triphosphates, depleted in deoxyadenosine triphosphate (referred to as dATP mix):

- 20 4μL of a 1.25 mM solution of dATP (Promega) are mixed with 4μL of 5 mM dTTP (Promega), 4μL of 5 mM dCTP (Promega) and 4 μL 5 mM dGTP (Promega).

Table 6

Preparation of the common mix for 15 reactions

	/reaction	/15 reactions
TACS buffer (x5)	2 μL	30 μL
dATP mix	1 μL	15 μL
Taq (5U/μL)	1 μL	15 μL
Template (Bluescript plasmid)	2 μL	30 μL

Preparation of the 2 mM stock solution of MATPP 7:

1.17 mg of MATPP 7 are diluted in 1.04 mL of H₂O.

5

Table 7

Reactions with 2 mM morpholino ATPP

	400 μM Morpholino ATPP	200 μM Morpholino ATPP	100 μM Morpholino ATPP
Common mix	6 μ L	6 μ L	6 μ L
Z1M13 Primer (JOE)	1 μ L	1 μ L	1 μ L
2 mM Morpholino ATPP	2 μ L	1 μ L	0.5 μ L
H ₂ O	1 μ L	2 μ L	2.5 μ L

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Table 8

Three control reactions with 2.5 mM dideoxyadenosine triphosphate (ddATP)

	250 μ M ddATP
Common mix	6 μ L
Z1M13 Primer (ROX)	1 μ L
2.5 mM ddATP	1 μ L
H ₂ O	2 μ L

Preparation of the 20 mM and 2 mM stock solutions of MATPPF 12

Solution S₀ at 20 mM: dilute the sample (2.2 mg) in 110.5 μ L of H₂O

Solution S₁ at 2 mM: take 10 μ L of S₀ and add 90 μ L of H₂O

Table 9

Reactions with 20 mM morpholino ATPPF (S₀)

	1000 μ M MATPPF	5000 μ M MATPPF
Common mix	6 μ L	6 μ L
Z1M13 Primer (JOE)	1 μ L	1 μ L
20 mM Morpholino ATPPF	0.5 μ L	2.5 μ L
H ₂ O	2.5 μ L	0.5 μ L

Table 10

Reactions with 2 mM morpholino ATPPF 2 mM (S₁)

	500 μ M MATPPF	200 μ M MATPPF
Common mix	6 μ L	6 μ L
Z1M13 Primer (JOE)	1 μ L	1 μ L
2 mM Morpholino ATPPF	2.5 μ L	1 μ L
H ₂ O	0.5 μ L	2 μ L

Table 11

Four control reactions with 2.5 mM dideoxyadenosine triphosphate (ddATP)

	ddATP 250 μ M
Common mix	6 μ L
Z1M13 Primer (ROX)	1 μ L
2.5 mM ddATP	1 μ L
H ₂ O	2 μ L

The results obtained with morpholino A putrescine **7** at 100 μ M and morpholino A fluorescein **12** at 5 mM, between the 90th and the 250th base, are given in Figure 2.

It is thus found that these two derivatives do indeed act as chain terminators. Furthermore, it should be noted that the reactions carried out with the fluorescent derivative, morpholino A fluorescein, were detected by means of the fluorophore borne by this

derivative: a fluorescent chain terminator was thus prepared.

Example 15: Use of morpholino A putrescine (MATPP) and morpholino A fluorescein (MATPPF) for the template-dependent 3' labelling of DNA fragments; test of enzymatic incorporation of these compounds by three polymerases (Taq, Klenow, Klenow Exo Free) and a reverse transcriptase.

These two nucleoside triphosphate derivatives are tested in enzymatic incorporation to label an oligonucleotide 13 bases long at its 3' end. This labelling is referred to as "template-dependent" since the enzymes used need the complementary strand to extend the oligonucleotide according to the Watson & Crick rules. Sequence A (17870 pmol/mL) studied and also its complementary target C (16128 pmol/mL) are given in the figure below:

20	Target C:	3'-TGC CAA CCA ACC CCA CCT CAA CCT CTG-5'
	Primer A:	5'-ACG GTT GGT TGG G (13 bp)
	Expected fragments:	5'-ACG GTT TGG GGT GGA (18 bp)
	and lengths (bp)	: 5'-ACG GTT GGT TGG GGT GGA GTT GGA (24 bp)
		5'-ACG GTT GGT TGG GGT GGA GTT GGA GA (26 bp)
25		5'-ACG GTT GGT TGG GGT GGA GTT GGA GAC (27 bp)

Three enzymes are used for this labelling: Taq DNA polymerase (Boehringer Mannheim), the Klenow fragment (Boehringer Mannheim) and the Klenow exonuclease-free polymerase (Amersham Life Science). The primer is labelled at its 5' end by incorporation of ^{32}P phosphate with the "Ready to go" T4 Polynucleotide Kinase kit (Pharmacia Biotech). The radiolabelled primer is noted A*.

The reaction buffers for the three enzymes are prepared for 10 reactions:

5

Table 12

(in μL)	Taq reaction	Klenow Exo Free reaction
C	50	50
A	10	10
A*	10	10
Tp 10X	50	50
H ₂ O	50	50

Table 13

(in μL)	Klenow reaction
C	50
A	10
A*	10
Tp 5X	100
H ₂ O	0

10

The enzymes are then diluted in the following manner, for 10 reactions:

- Taq (5U/ μL): 10X0.1 μL of Taq + 10X15.5 μL of H₂O
- 15 - Klenow (20U/ μL): 10X0.1 μL of Klenow + 10X15.5 μL of H₂O
- Klenow Exo Free (5U/ μL): 10X0.1 μL of Klenow Exo Free + 10X15.5 μL of H₂O.

Solutions containing the normal nucleoside triphosphates are also prepared:

- Solution "2P" composed of a mixture of dGTP and dTTP each at 0.1 mM

20

- The implementation reactions are described in Table 14 below:

Table 14

(in μL)	1	2	3	4	5	6	7	8	9
		400 μM	200 μM	50 μM	2.5 mM	400 μM	200 μM	50 μM	
Reaction	17	17	17	17	17	17	17	17	17
"2p"	0	5	5	5	5	5	5	5	0
"4p"	0	0	0	0	0	0	0	0	5
2 mM MATPP	0	10	5	1,25	0	0	0	0	0
20 mM MATPPF	0	0	0	0	6.25	0	0	0	0
2 mM MATPPF	0	0	0	0	0	10	5	1.25	0
H ₂ O	33	2.7	7.4	11.15	6.15	2.4	7.4	11.15	12.4
Enzyme	0	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6

The morpholino A putrescine is thus tested at three concentrations: 400, 200 and 50 μ M, while the morpholino A fluorescein is reacted at 2.5 mM, 400, 200 and 50 μ M.

5 Before adding the enzyme, the mixture is denatured at 94°C for 5 minutes. It is then left to return to ambient temperature in order for the hybridization to take place. The elongation is carried out at 70°C for the Taq and at 37°C for the two Klenow
10 fragments, and for 10 minutes. Finally, the medium is again denatured with a formamide solution and heating at 90°C for 5 minutes, after which it is placed on a polyacrylamide gel. The separation is carried out by electrophoresis at 2 000 V. The gel is read using a
15 Phosphorimager; the results obtained are given in Figure 3.

In this figure, the lanes 1 serve as migration control for the labelled oligonucleotide A. This oligonucleotide has a length of 13 bases (13-mer).
20 Lanes 2, 3 and 4 allow the elongation of the oligonucleotide A and the incorporation of the morpholino A putrescine to be monitored. Under these conditions, only the nucleotides dGTP and dTTP (solution "2P") were added and can be used by the
25 enzyme to carry out the extension of the primer. The presence of the morpholino A putrescine in the reaction medium allows its incorporation at the level of base 18. A control was carried out, placing in the medium only the "2P" mixture; in this case, the enzyme
30 continues its extension up to the 17th base since it has no adenosine derivative to continue its polymerization. Thus, the difference in migration between this control, which is 17 bases long, and

reactions 2, 3 and 4 confirms the incorporation of MATPP and the interruption of the elongation of the chain. Reactions 5 to 8 correspond to the same reactions with morpholino A fluorescein. Here also, the MATPPF is indeed incorporated and stops the polymerization of the complementary strand. It is noted, however, for the two Klenow fragments, that there was occasionally incorporation of another base (G or T) in place of the morpholino derivative. Specifically, in these cases, elongation products corresponding to the 18-mer and 24-mer are found.

Well 9 (see Figure 4) is a control reaction: the reaction medium contains the 4 normal deoxynucleotides and can consequently extend the primer up to its maximum extension, that is to say until the 27-mer is obtained.

In conclusion, the three enzymes incorporate the morpholino A putrescine and morpholino A fluorescein in all the concentrations tested, including the weakest concentrations.

The capacity of the reverse transcriptases to incorporate the morpholino nucleotide derivatives in the course of the extension of oligonucleotides was confirmed. In this test, the reverse transcriptase (M-MLV, Promega; activity: 200 000 U/mL) is chosen as model. This enzyme is capable of synthesizing a DNA strand complementary to a target strand (DNA or RNA), from an oligonucleotide primer, in the presence of nucleoside triphosphates. Morpholino A putrescine and morpholino A fluorescein are thus tested at final concentrations of 250 μ M. A control copy is also deposited on the gel, with the four nucleoside triphosphates of the "4P" solution.

The sequence of the target C (27-mer, 16128 pmol/mL) and that of the primer B (14-mer, 56368 pmol/mL) are shown below. This primer B, which is radioactively labelled, is noted B*.

5 The solution B* thus contains 10 pmol of primer B in a volume of 50 μ L. The solutions of C and B are also diluted tenfold; these solutions are noted, respectively, C/10 and B/10.

10 Target C : 3'-TGC CAA CCA ACC CCA CCT CAA CCT CTG-5'
Primer B : 5'-ACG GTT GGT TGG GG (14 bp)

Table 15

15

(in μ L)	Reaction 1	Reaction 2	Reaction 3	Reaction 4
C/10	2	2	2	0
B*	5	5	5	5
B/10	3	3	3	0
5X Buffer	4	4	4	0
2 mM MATPP	2.5	0	0	0
2 mM MATPPF	0	2.5	0	0
"2P"	2.5	2.5	0	0
"4P"	0	0	2.5	0
H ₂ O	0	0	0	15
Enzyme	1	1	1	0

As previously, the mixture is denatured, before adding the enzyme, at 94°C for 5 minutes and is left to cool to ambient temperature. The elongation is carried out at 37°C for 60 minutes. The medium is denatured with a formamide solution and heating at 90°C for 5 minutes before being deposited on a polyacrylamide gel. The separation is carried out by electrophoresis at 1500 V. The gel is read using a

20

Phosphorimager; the results obtained are given in Figure 4.

In this figure, lane 4 allows the length of the labelled primer B to be estimated. Lane 3 shows the maximum elongation of the primer B up to a final product of 27 base pairs in the presence of the four natural deoxynucleotides. Reactions 1 and 2 show that the morpholino derivatives are incorporated in the course of the elongation of the primer B with the reverse transcriptase. This incorporation is quantitative and gives a product of 18 base pairs (in the absence of morpholino derivative, the extension is blocked at the 17th base).

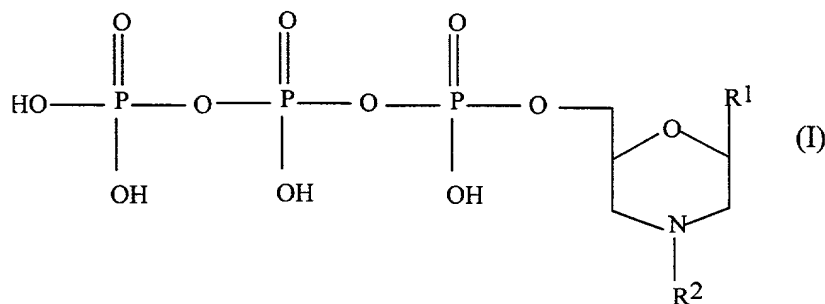
In conclusion, the morpholino derivatives are very well recognized by reverse transcriptase and incorporated into the primers during extension in a base-specific process.

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- [1] : Sanger et al., Proceedings of National Academy of Science, 74, 1977, p. 5463-5467.
- [2] : WO-A-96/23807.
- [3] : Prober et al., Science, 238, 1987, pages 336-341.
- [4] : Hileman et al., Bioconjugate Chemistry, 5, 1994, pages 436-444.
- [5] : Broker et al., Nucleic Acids Research, 5, 1978, pages 363-385.
- [6] : Agrawal et al., Nucleic Acids Research, 14, 1986, pages 6227-6245.
- [7] : FR-A-2 710 068
- [8] : Rayford et al., Journal of Biological Chemistry, 260, 1985, pages 15708-15713.

CLAIMS

1. Process for manufacturing a 3'-labelled nucleic acid (DNA or RNA) fragment, which comprises the enzymatic incorporation of a nucleotide derivative having as precursor a compound of formula:



- in which R^1 represents a nucleic base and R^2 represents a group corresponding to one of the following formulae:

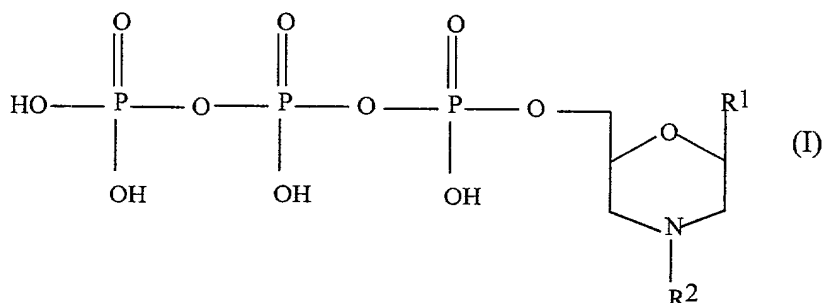


in which n is an integer ranging from 1 to 12 and R^3 is a group derived from a label, a protein, an enzyme, a fatty acid or a peptide, at the 3' OH end of the nucleic acid fragment.

2. Process for modifying a nucleic acid fragment by enzymatic incorporation at the 3' end of a modified morpholino nucleotide having as precursor a compound corresponding to the formula:

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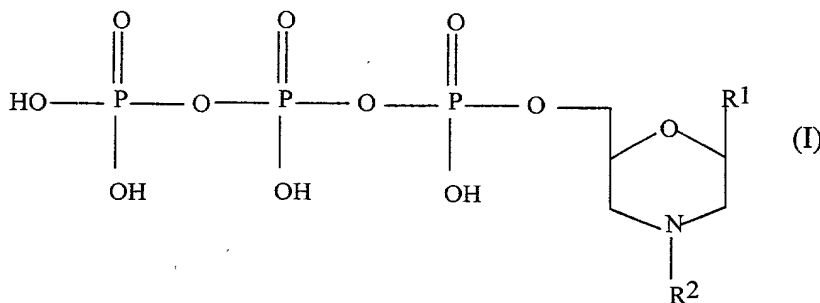


in which R^1 represents a nucleic base and R^2 represents
a group corresponding to one of the following formulae:

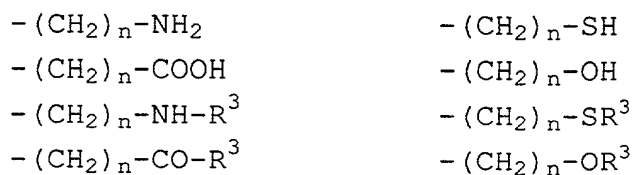
- $-(CH_2)_n-NH-R^3$
- $-(CH_2)_n-CO-R^3$
- $-(CH_2)_n-SR^3$
- $-(CH_2)_n-OR^3$

in which n is an integer ranging from 1 to 12 and R^3
represents a compound chosen from photo-crosslinking
agents, fatty acids, hydrophobic peptides, antibodies,
enzymes and fluorophores.

3. Process for sequencing a nucleic acid
(DNA or RNA) by the technique of enzymatic
polymerization of the sequence complementary to this
nucleic acid using chain terminators, in which at least
one of the chain terminators has as precursor a
compound corresponding to the formula:



in which R^1 represents a nucleic base and R^2 represents
a group corresponding to one of the following formulae:



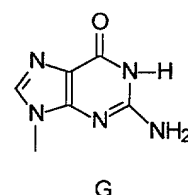
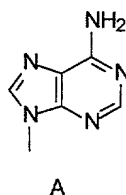
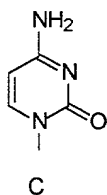
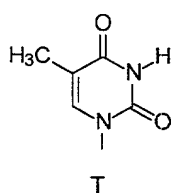
5 in which n is an integer ranging from 1 to 12 and R^3 is a group derived from a label, a protein, an enzyme, a fatty acid or a peptide.

10 4. Process according to Claim 1, 2 or 3, in which the enzyme is the Klenow fragment of DNA polymerase.

15 5. Process according to Claim 1, 2 or 3, in which the enzyme is a heat-resistant polymerase of a *Thermophilus* bacterium or terminal transferase or reverse transcriptase.

20 6. Process according to one of Claims 1 to 5, in which the nucleic base is a natural nucleic base chosen from adenine, guanine, cytosine, thymine, uracil, xanthine, hypoxanthine and 2-aminopurine, and derivatives thereof.

25 7. Process according to any one of Claims 1 to 5, in which R^1 corresponds to one of the following formulae:



30 8. Process according to one of Claims 1 to 7, in which the label is chosen from radioactive products, luminescent products, electroluminescent and

fluorescent products, molecules capable of coupling with other molecules, molecules which allow interactions of the antigen-antibody type, and enzymatic labels.

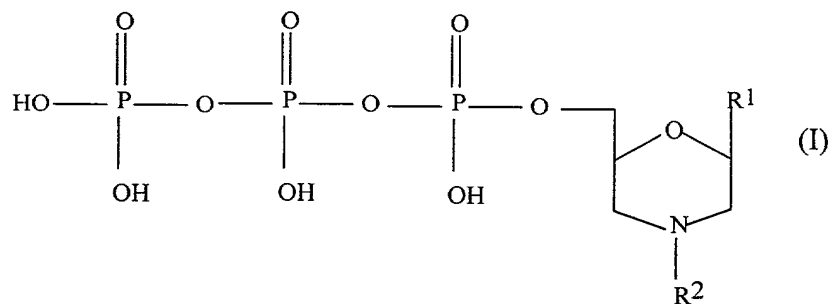
5

9. Process according to any one of the claims 1 to 7, in which R^3 is a fluorophore.

10. Process according to Claim 9, in which R^3 is chosen from fluorescein derivatives, biotin derivatives and rhodamine derivatives.

11. Process according to Claim 1, 2 or 3, in which the derivative, the modified morpholino-nucleotide or the chain terminator is compound (I) in monophosphate form.

12. Morpholino-nucleotide corresponding to the formula:

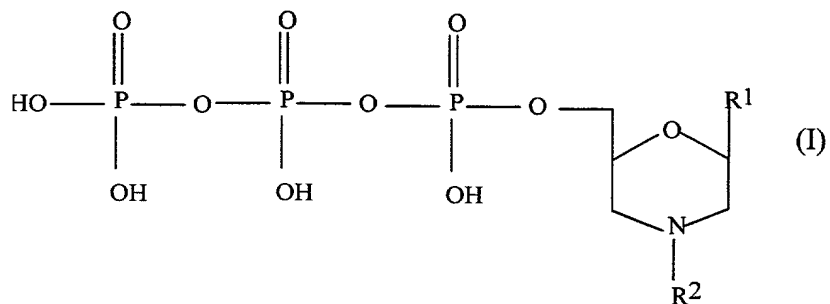


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in which R^1 is adenine and R^2 represents $-\text{CH}_2-\text{COOH}$, $-(\text{CH}_2)_4-\text{NH}_2$ or $-(\text{CH}_2)_4-\text{NH}-\text{R}^3$ with R^3 representing a group derived from fluorescein.

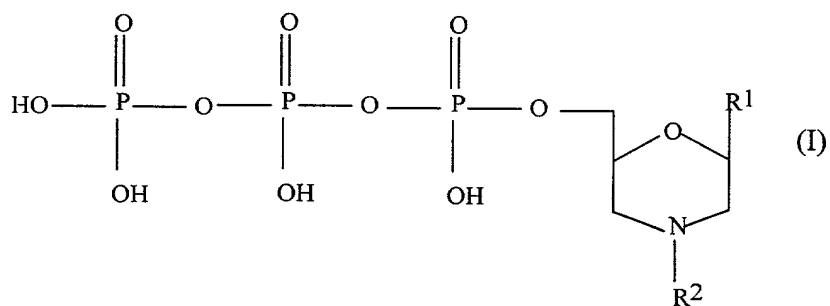
25

13. Morpholino-nucleotide of formula:



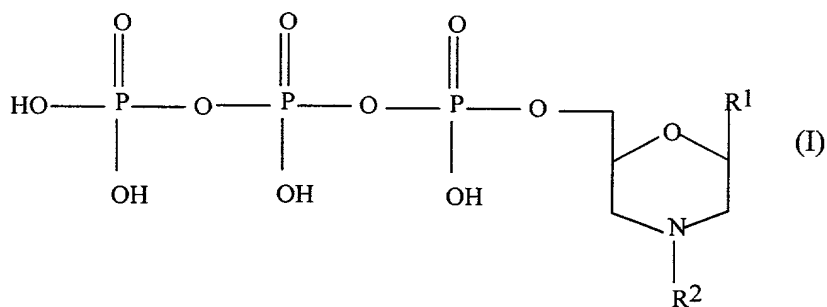
in which R^1 is thymine and R^2 represents $-\text{CH}_2-\text{COOH}$,
 $-(\text{CH}_2)_4-\text{NH}_2$ or $-(\text{CH}_2)_4-\text{NH}-\text{R}^3$ with R^3 representing a group
 5 derived from fluorescein.

14. Morpholino-nucleotide corresponding to
 the formula:



10 in which R^1 is cytosine and R^2 represents $-\text{CH}_2-\text{COOH}$,
 $-(\text{CH}_2)_4-\text{NH}_2$ or $-(\text{CH}_2)_4-\text{NH}-\text{R}^3$ with R^3 representing a group
 derived from fluorescein.

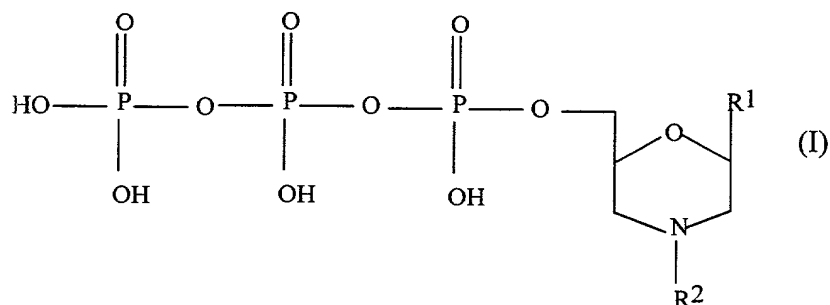
15 15. Morpholino-nucleotide corresponding to
 the formula:



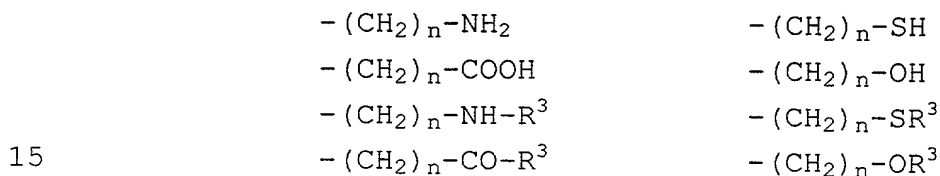
in which R^1 is guanine and R^2 represents $-\text{CH}_2-\text{COOH}$, $-(\text{CH}_2)_4-\text{NH}_2$ or $-(\text{CH}_2)_4-\text{NH}-R^3$ with R^3 representing a group derived from fluorescein.

5

16. Process for manufacturing a morpholino-nucleotide of formula:



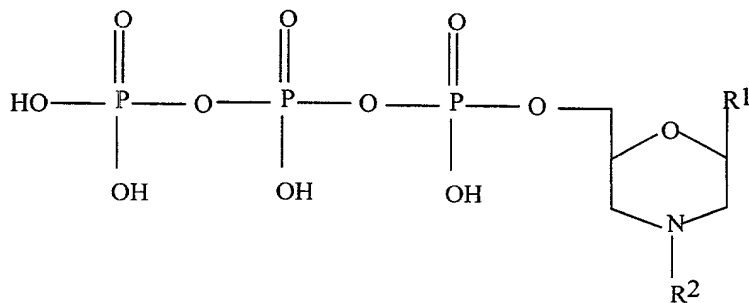
10 in which R^1 represents a nucleic base and R^2 represents a group corresponding to one of the following formulae:



15

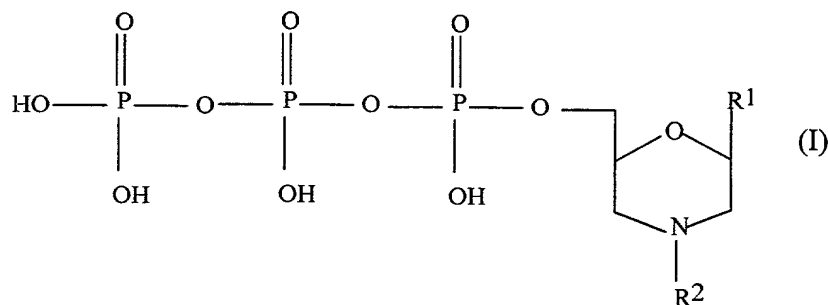
in which n is an integer ranging from 1 to 12 and R^3 is a group derived from a label, from a protein, from an enzyme, from a fatty acid or from a peptide, the reaction of a nucleoside triphosphate of formula:

20



in which R^1 has the meaning given above, with a periodate, a compound of formula $R^2 NH_2$ in which R^2 has the meaning given above, and sodium borohydride.

- 5 17. Use of a morpholino-nucleotide of formula:

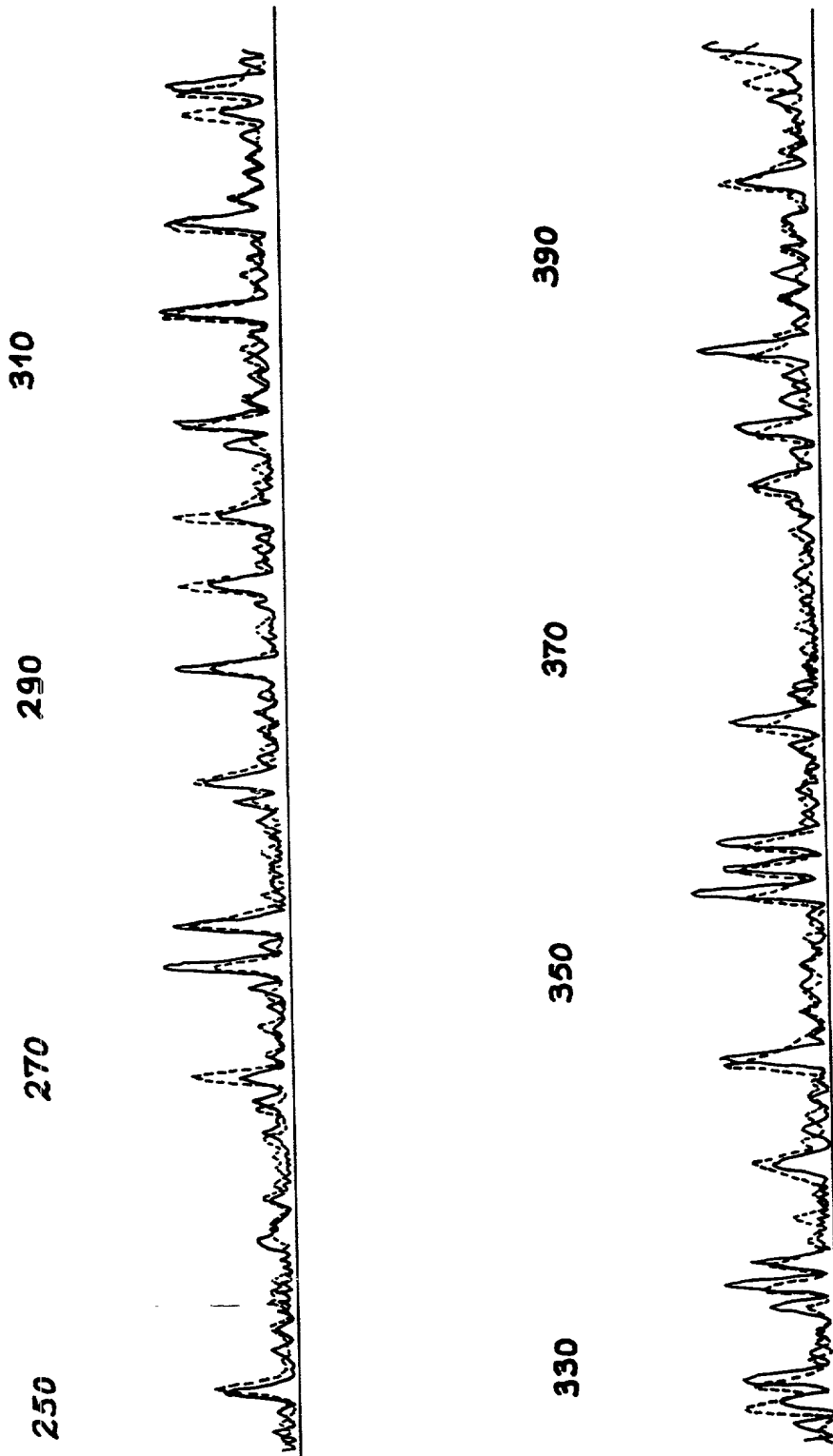


in which R^1 represents a nucleic base and R^2 represents a group corresponding to one of the following formulae:

- 10 $-(CH_2)_n-NH_2$ $-(CH_2)_n-SH$
 $-(CH_2)_n-COOH$ $-(CH_2)_n-OH$
 $-(CH_2)_n-NH-R^3$ $-(CH_2)_n-SR^3$
 $-(CH_2)_n-CO-R^3$ $-(CH_2)_n-OR^3$

- 15 in which n is an integer ranging from 1 to 12 and R^3 is a group derived from a label, from a protein, from an enzyme, from a fatty acid or from a peptide, for the labelling of DNA or RNA fragments.

FIG. 1



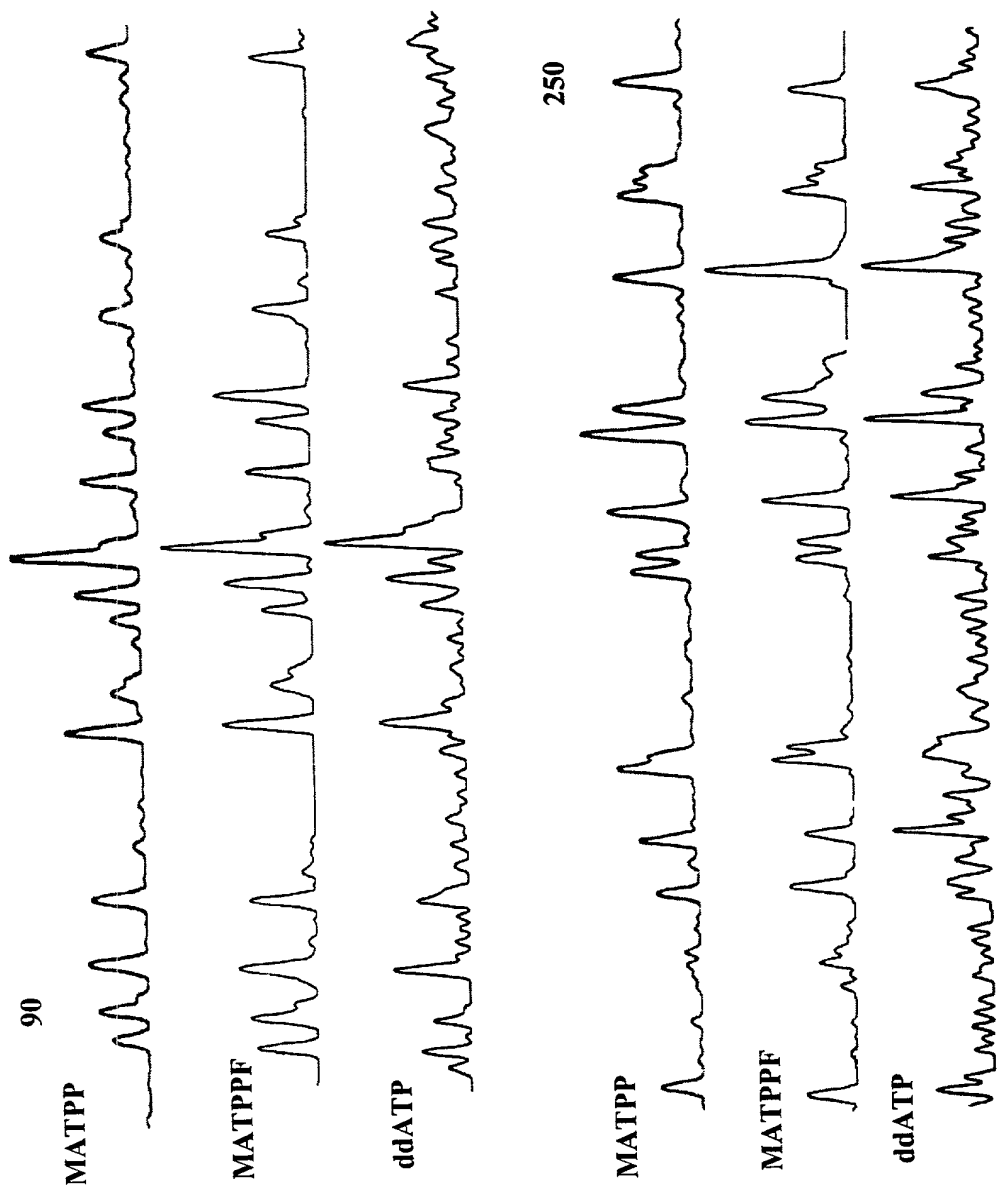


FIG. 2

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FIG. 3

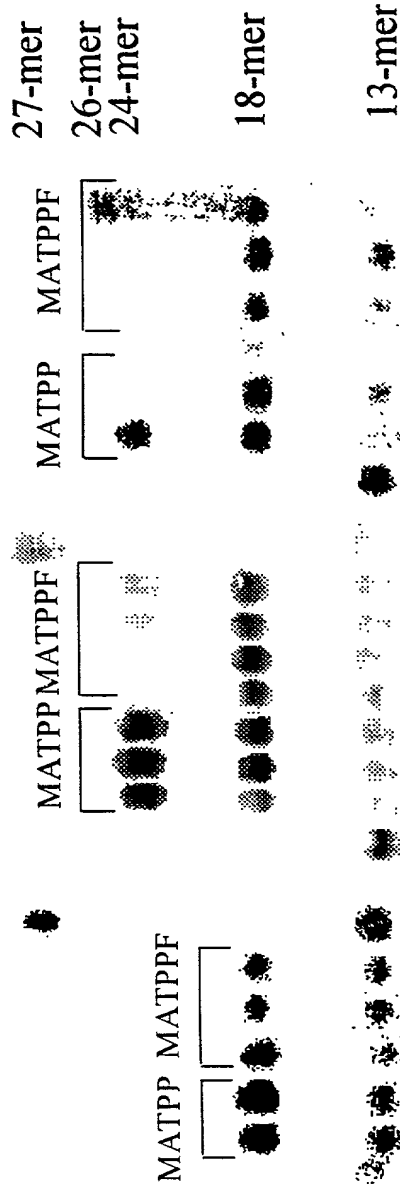
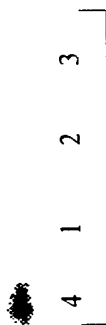
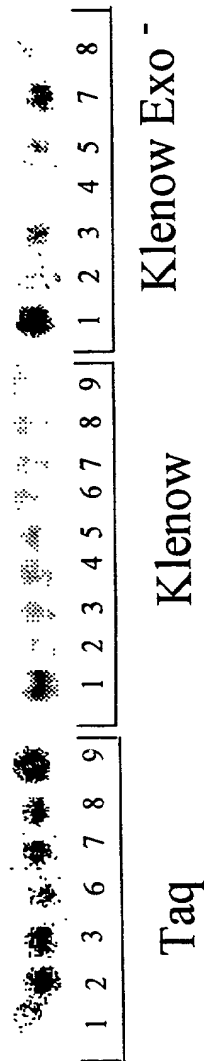


FIG. 4



Klenow Exo -

Klenow

Taq

Declaration, Power Of Attorney and Petition

Page 1 of 3

WE (I) the undersigned inventor(s), hereby declare(s) that :

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

PROCESS FOR MANUFACTURING MORPHOLINO-NUCLEOTIDES, AND USE THEREOF FOR THE ANALYSIS OF AND LABELLING OF NUCLEIC ACID SEQUENCES

the specification of which

- ☐ is attached hereto.
- ☐ was filed on
as Application Serial No.
and amended on
- ☒ was filed as PCT international application
Number PCT/FR00/00427
on February 21, 2000
and was amended under PCT Article 19
on January 24, 2001

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119 (a)-(d) or § 365 (b) of any foreign application(s) for patent or inventor's certificate, or § 365 (a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application (s)

Application No.	Country	Day/month/Year	Priority Claimed
99 02170	FRANCE	22 FEBRUARY 1999	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
99 12001	FRANCE	27 SEPTEMBER 1999	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

We (I) hereby claim the benefit under Title 35, United States Code, § 119 (e) of any United States provisional application(s) listed below.

(Application Number)

(Filing Date)

(Application Number)

(Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. §120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of prior application and the national or PCT International filing date of this application.

Status (pending, patented,

Application Serial No.

Filing Date


abandoned)

And we (I) hereby appoint :William L. Mathis, Registration Number 17,337; Alan E. Kopecki, Registration Number 25,813; Eric H. Weisblatt, Registration Number 30,505; Peter H. Smolka, Registration Number 15,913; Regis E. Slutter, Registration Number 26,999; James W. Peterson, Registration Number 26,057; Robert S. Swecker, Registration Number 19,885; Samuel C. Miller III, Registration Number 27,360; Terasa Stanek REA, Registration Number 30,427; Platon N. Mandros, Registration Number 22,124; Ralph L. Freeland Jr., Registration Number 16,110; Robert E. Krebs, Registration Number 25,885; Benton S. Duffett jr., Registration Number 22,030; Robert M. Schulman, Registration Number 31,196; Joel M. Freed, Registration Number 25,101; James A. Labarre, Registration Number 28,632; William C. Rowland, Registration Number 30,888; Norman H. Stepno, Registration Number 22,716; E. Joseph Gess, Registration Number 28,510; Richard H. Kjeldgaard, Registration Number 30,186; Ronald L. Grudziecki, Registration Number 24,970; David D. Reynolds, Registration Number 29,273; T. Gene Dillahunty, Registration Number 25,423; Frederick G. Michaud Jr, Registration Number 26,003; R. Danny Huntington, Registration Number 27,903 and Anthony W. Shaw, Registration Number 30,104; our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to the firm of BURNS, DOANE, SWECKER & MATHIS, whose post Office Address is : George Mason Building, Washington and Prince Streets, P.O. Box 1404 Alexandria, Virginia 22313-1404

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true ; and future that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of the application or any patent issuing thereon.

MARCIACQ Florence

NAME OF FIRST SOLE INVENTOR



Signature of Inventor

August 03, 2001
Date

Residence : 6 rue GAMBETTA
13580 LA FARE LES OLIVIERS
FRANCE

Citizen of : FRANCE

Post Office Address : The same as residence

Signature of Inventor

August 03, 2001

Date _____

Residence: Le Noyaret

38320 Herbey
FRANCE ~~FR~~

Citizen of: FRANCE

Post Office Address : The same as residence

MOURET Jean-François

NAME OF THIRD INVENTOR

Signature of Inventor

August 03, 2001

Date _____

Residence : Pontée du Pilet

38500 COUBLEVIE
FRANCE FAX

Citizen of: France

Post Office Address : The same as residence

ISSARTEL Jean-Paul

NAME OF FOURTH INVENTOR

Signature of Inventor

August 03, 2001

Date _____

Residence : 9 rue du FOURNET

38120 ST ECREVE FRANCE F11X

Citizen of: FRANCE

Post Office Address : The same as residence

MOLKO Didier

NAME OF FIFTH INVENTOR

Signature of Inventor

August 03, 2001

Date _____

Residence : Les Noyers A1-1

38210 Tullins - France FLX

Citizen of: FRANCE

Post Office Address : The same as residence